



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07H 21/04, C07K 14/00, C12N 15/00	A1	(11) International Publication Number: WO 00/55178 (43) International Publication Date: 21 September 2000 (21.09.00)
(21) International Application Number: PCT/US00/06602 (22) International Filing Date: 13 March 2000 (13.03.00) (30) Priority Data: 09/268,969 16 March 1999 (16.03.99) US 60/184,373 23 February 2000 (23.02.00) US (71) Applicant: EXELIXIS, INC. [US/US]; 280 East Grand Avenue, South San Francisco, CA 94080 (US). (72) Inventors: BUCHMAN, Andrew, Roy; 3119 Epton Avenue, Berkeley, CA 94705 (US). PLATT, Darren, Mark; 929 Pine Street, Apt. 201, San Francisco, CA 94108 (US). OLLMAN, Michael, Martin; 1805 Atschul Avenue, Menlo Park, CA 94025 (US). YOUNG, Lynn, Marie; 250 Baldwin Avenue, #4, San Mateo, CA 94401 (US). DEMSKY, Madelyn, Robin; 1770 Pine Street, 3203, San Francisco, CA 94109 (US). KEEGAN, Kevin, Patrick; 17311 Via Estrella, San Lorenzo, CA 94580 (US). FRIEDMAN, Lori; One Bayside Village Place, Unit 212, San Francisco, CA 94107 (US). KOPCZYNSKI, Casey; 2769 St. James Road, Belmont, CA 94002 (US). LARSON, Jeffrey, S.; 1220 El Camino Real #305, Burlingame, CA 94010 (US). ROBERTSON, Stephanie, A.; 255 Fowler Avenue, San Francisco, CA 94127 (US).		(74) Agent: BRUNELLE, Jan, P.; Exelixis, Inc., 280 East Grand Avenue, South San Francisco, CA 94080 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: INSECT p53 TUMOR SUPPRESSOR GENES AND PROTEINS (57) Abstract A family of p53 tumor suppressor nucleic acid and protein isolated from several insect species is described. The p53 nucleic acid and protein can be used to genetically modify metazoan invertebrate organisms, such as insects and worms, or cultured cells, resulting in p53 expression or mis-expression. The genetically modified organisms or cells can be used in screening assays to identify candidate compounds that are potential pesticidal agents or therapeutics that interact with p53 protein. They can also be used in methods for studying p53 activity and identifying other genes that modulate the function of, or interact with, the p53 gene. Nucleic acid and protein sequences for <i>Drosophila</i> p33 and Rb tumor suppressors are also described.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INSECT p53 TUMOR SUPPRESSOR GENES AND PROTEINS

REFERENCE TO RELATED APPLICATION

5 This application is a continuation-in-part of U.S. application no. 09/268,969, filed March 16, 1999; and of U.S. application no. 60/184,373 of same title, filed February 23, 2000. The entire contents of both prior applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10 The p53 gene is mutated in over 50 different types of human cancers, including familial and spontaneous cancers, and is believed to be the most commonly mutated gene in human cancer (Zambetti and Levine, FASEB (1993) 7:855-865; Hollstein, *et al.*, Nucleic Acids Res. (1994) 22:3551-3555). Greater than 90% of mutations in the p53 gene are missense mutations that alter a single amino acid that inactivates p53 function. Aberrant
15 forms of human p53 are associated with poor prognosis, more aggressive tumors, metastasis, and survival rates of less than 5 years (Koshland, Science (1993) 262:1953).

 The human p53 protein normally functions as a central integrator of signals arising from different forms of cellular stress, including DNA damage, hypoxia, nucleotide deprivation, and oncogene activation (Prives, Cell (1998) 95:5-8). In response to these
20 signals, p53 protein levels are greatly increased with the result that the accumulated p53 activates pathways of cell cycle arrest or apoptosis depending on the nature and strength of these signals. Indeed, multiple lines of experimental evidence have pointed to a key role for p53 as a tumor suppressor (Levine, Cell (1997) 88:323-331). For example, homozygous p53 "knockout" mice are developmentally normal but exhibit nearly 100% incidence of
25 neoplasia in the first year of life (Donehower *et al.*, Nature (1992) 356:215-221). The biochemical mechanisms and pathways through which p53 functions in normal and cancerous cells are not fully understood, but one clearly important aspect of p53 function is its activity as a gene-specific transcriptional activator. Among the genes with known p53-response elements are several with well-characterized roles in either regulation of the cell
30 cycle or apoptosis, including GADD45, p21/Waf1/Cip1, cyclin G, Bax, IGF-BP3, and MDM2 (Levine, Cell (1997) 88:323-331).

Human p53 is a 393 amino acid phosphoprotein which is divided structurally and functionally into distinct domains joined in the following order from N-terminus to C-terminus of the polypeptide chain: (a) a transcriptional activation domain; (b) a sequence-specific DNA-binding domain; (c) a linker domain; (d) an oligomerization domain; and (e) a basic regulatory domain. Other structural details of the p53 protein are in keeping with its function as a sequence-specific gene activator that responds to a variety of stress signals. For example, the most N-terminal domain of p53 is rich in acidic residues, consistent with structural features of other transcriptional activators (Fields and Jang, Science (1990) 249:1046-49). By contrast, the most C-terminal domain of p53 is rich in basic residues, and has the ability to bind single-stranded DNA, double-stranded DNA ends, and internal deletions loops (Jayaraman and Prives, Cell (1995) 81: 1021-1029). The association of the p53 C-terminal basic regulatory domain with these forms of DNA that are generated during DNA repair may trigger conversion of p53 from a latent to an activated state capable of site-specific DNA binding to target genes (Hupp and Lane, Curr. Biol. (1994) 4: 865-875), thereby providing one mechanism to regulate p53 function in response to DNA damage. Importantly, both the N-terminal activation domain and the C-terminal basic regulatory domain of p53 are subject to numerous covalent modifications which correlate with stress-induced signals (Prives, Cell (1998) 95:5-8). For example, the N-terminal activation domain contains residues that are targets for phosphorylation by the DNA-activated protein kinase, the ATM kinase, and the cyclin activated kinase complex. The C-terminal basic regulatory domain contains residues that are targets for phosphorylation by protein kinase C, cyclin dependent kinase, and casein kinase II, as well as residues that are targets for acetylation by PCAF and p300 acetyl transferases. p53 activity is also modulated by specific non-covalent protein-protein interactions (Ko and Prives, Genes Dev. (1996) 10: 1054-1072). Most notably, the MDM2 protein binds a short, highly conserved protein sequence motif, residues 13-29, in the N-terminal activation domain of p53 (Kussie *et al.*, Science (1996) 274:948-953. As a result of binding p53, MDM2 both represses p53 transcriptional activity and promotes the degradation of p53.

Although several mammalian and vertebrate homologs of the tumor suppressor p53 have been described, only two invertebrate homologs have been identified to date in mollusc and squid. Few lines of evidence, however, have hinted at the existence of a p53 homolog in any other invertebrate species, such as the fruit fly *Drosophila*. Indeed, numerous direct attempts to isolate a *Drosophila* p53 gene by either cross-hybridization or PCR have failed to identify a p53-like gene in this species (Soussi *et al.*, Oncogene (1990)

5: 945-952). However, other studies of response to DNA damage in insect cells using nucleic cross-hybridization and antibody cross-reactivity have provided suggestive evidence for existence of p53-, p21-, and MDM2-like genes (Bae *et al.*, Exp Cell Res (1995) 375:105-106; Yakes, 1994, Ph.D. thesis, Wayne State University). Nonetheless, no isolated insect p53 genes or proteins have been reported to date.

Identification of novel p53 orthologues in model organisms such as *Drosophila melanogaster* and other insect species provides important and useful tools for genetic and molecular study and validation of these molecules as potential pharmaceutical and pesticide targets. The present invention discloses insect p53 genes and proteins from a variety of diverse insect species. In addition, *Drosophila* homologs of p33 and Rb genes, which are also involved in tumor suppression, are described.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide insect p53 nucleic acid and protein sequences that can be used in genetic screening methods to characterize pathways that p53 may be involved in as well as other interacting genetic pathways. It is also an object of the invention to provide methods for screening compounds that interact with p53 such as those that may have utility as therapeutics.

These and other objects are provided by the present invention which concerns the identification and characterization of insect p53 genes and proteins in a variety of insect species. Isolated nucleic acid molecules are provided that comprise nucleic acid sequences encoding p53 polypeptides and derivatives thereof. Vectors and host cells comprising the p53 nucleic acid molecules are also described, as well as metazoan invertebrate organisms (*e.g.* insects, coelomates and pseudocoelomates) that are genetically modified to express or mis-express a p53 protein.

An important utility of the insect p53 nucleic acids and proteins is that they can be used in screening assays to identify candidate compounds which are potential therapeutics or pesticides that interact with p53 proteins. Such assays typically comprise contacting a p53 polypeptide with one or more candidate molecules, and detecting any interaction between the candidate compound and the p53 polypeptide. The assays may comprise adding the candidate molecules to cultures of cells genetically engineered to express p53 proteins, or alternatively, administering the candidate compound to a metazoan invertebrate organism genetically engineered to express p53 protein.

The genetically engineered metazoan invertebrate animals of the invention can also be used in methods for studying p53 activity, or for validating therapeutic or pesticidal strategies based on manipulation of the p53 pathway. These methods typically involve detecting the phenotype caused by the expression or mis-expression of the p53 protein. The methods may additionally comprise observing a second animal that has the same genetic modification as the first animal and, additionally has a mutation in a gene of interest. Any difference between the phenotypes of the two animals identifies the gene of interest as capable of modifying the function of the gene encoding the p53 protein.

BRIEF DESCRIPTION OF THE FIGURE

Figures 1A-1B show a CLUSTALW alignment of the amino acid sequences of the insect p53 proteins identified from *Drosophila*, *Leptinotarsa*, *Tribolium*, and *Heliothis*, with p53 sequences previously identified in human, *Xenopus*, and squid. Identical amino acid residues within the alignment are grouped within solid lines and similar amino acid residues are grouped within dashed lines.

DETAILED DESCRIPTION OF THE INVENTION

The use of invertebrate model organism genetics and related technologies can greatly facilitate the elucidation of biological pathways (Scangos, Nat. Biotechnol. (1997) 15:1220-1221; Margolis and Duyk, Nature Biotech. (1998) 16:311). Of particular use is the insect model organism, *Drosophila melanogaster* (hereinafter referred to generally as "*Drosophila*"). An extensive search for p53 nucleic acid and its encoded protein in *Drosophila* was conducted in an attempt to identify new and useful tools for probing the function and regulation of the p53 genes, and for use as targets in drug discovery. p53 nucleic acid has also been identified in the following additional insect species: *Leptinotarsa decemlineata* (Colorado potato beetle, hereinafter referred to as *Leptinotarsa*), *Tribolium castaneum* (flour beetle, hereinafter referred to as *Tribolium*), and *Heliothis virescens* (tobacco budworm, hereinafter referred to as *Heliothis*).

The newly identified insect p53 nucleic acids can be used for the generation of mutant phenotypes in animal models or in living cells that can be used to study regulation of p53, and the use of p53 as a drug or pesticide target. Due to the ability to rapidly carry out large-scale, systematic genetic screens, the use of invertebrate model organisms such as *Drosophila* has great utility for analyzing the expression and mis-expression of p53 protein. Thus, the invention provides a superior approach for identifying other components involved

in the synthesis, activity, and regulation of p53 proteins. Systematic genetic analysis of p53 using invertebrate model organisms can lead to the identification and validation of compound targets directed to components of the p53 pathway. Model organisms or cultured cells that have been genetically engineered to express p53 can be used to screen candidate compounds for their ability to modulate p53 expression or activity, and thus are useful in the identification of new drug targets, therapeutic agents, diagnostics and prognostics useful in the treatment of disorders associated with cell cycle, DNA repair, and apoptosis. The details of the conditions used for the identification and/or isolation of insect p53 nucleic acids and proteins are described in the Examples section below. Various non-limiting embodiments of the invention, applications and uses of the insect p53 genes and proteins are discussed in the following sections. The entire contents of all references, including patent applications, cited herein are incorporated by reference in their entireties for all purposes. Additionally, the citation of a reference in the preceding background section is not an admission of prior art against the claims appended hereto.

p53 Nucleic Acids

The following nucleic acid sequences encoding insect p53 are described herein: SEQ ID NO:1, isolated from *Drosophila*, and referred to herein as DMp53; SEQ ID NO:3, isolated from *Leptinotarsa*, and referred to herein as CPBp53; SEQ ID NO:5 and SEQ ID NO:7, isolated from *Tribolium*, and referred to herein as TRIB-Ap53 and TRIB-Bp53, respectively; and SEQ ID NO:9, isolated from *Heliothis*, and referred to herein as HELIOp53. The genomic sequence of the DMp53 gene is provided in SEQ ID NO:18.

In addition to the fragments and derivatives of SEQ ID NOs:1, 3, 5, 7, 9, and 18, as described in detail below, the invention includes the reverse complements thereof. Also, the subject nucleic acid sequences, derivatives and fragments thereof may be RNA molecules comprising the nucleotide sequences of SEQ ID NOs:1, 3, 5, 7, 9, and 18 (or derivative or fragment thereof) wherein the base U (uracil) is substituted for the base T (thymine). The DNA and RNA sequences of the invention can be single- or double-stranded. Thus, the term "isolated nucleic acid sequence" or "isolated nucleic acid molecule", as used herein, includes the reverse complement, RNA equivalent, DNA or RNA single- or double-stranded sequences, and DNA/RNA hybrids of the sequence being described, unless otherwise indicated.

Fragments of the p53 nucleic acid sequences can be used for a variety of purposes. Interfering RNA (RNAi) fragments, particularly double-stranded (ds) RNAi, can be used to

generate loss-of-function phenotypes. p53 nucleic acid fragments are also useful as nucleic acid hybridization probes and replication/amplification primers. Certain "antisense" fragments, i.e. that are reverse complements of portions of the coding sequence of any of SEQ ID NO:1, 3, 5, 7, 9, or 18 have utility in inhibiting the function of p53 proteins. The fragments are of length sufficient to specifically hybridize with the corresponding SEQ ID NO:1, 3, 5, 7, 9, or 18. The fragments consist of or comprise at least 12, preferably at least 24, more preferably at least 36, and more preferably at least 96 contiguous nucleotides of any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18. When the fragments are flanked by other nucleic acid sequences, the total length of the combined nucleic acid sequence is less than 15 kb, preferably less than 10 kb or less than 5kb, more preferably less than 2 kb, and in some cases, preferably less than 500 bases. Preferred p53 nucleic acid fragments comprise regulatory elements that may reside in the 5' UTR and/or encode one or more of the following domains: an activation domain, a DNA binding domain, a linker domain, an oligomerization domain, and a basic regulatory domain. The approximate locations of these regions in SEQ ID Nos 1, 3, and 5, and in the corresponding amino acid sequences of SEQ ID Nos 2, 4, and 6, 8, are provided in Table 1.

TABLE 1

	SEQ ID NOs		
	1/2	3/4	5/6
Insect Genus	<i>Drosophila</i>	<i>Leptinotarsa</i>	<i>Tribolium</i>
5' UTR	na 1-111	na 1-120	na 1-93
Activation Domain	na 112-257 aa 1-48	na 121-300 aa 1-60	na 94-277 aa 1-60
DNA Binding Domain	na 366-954 aa 85-280	na 321-936 aa 67-271	na 280-892 aa 62-265
Linker Domain	na 999-1056 aa 296-314	na 937-999 aa 272-292	na 893-958 aa 266-287
Oligomerization Domain	na 1065-1170 aa 318-352	na 1000-1113 aa 293-330	na 959-1075 aa 288-326
Basic Regulatory Domain	na 1179-1269 aa 356-385	na 1114-1182 aa 331-353	na 1076-1147 aa 327-350

Further preferred are fragments of bases 354-495 of SEQ ID NO:7 and bases 315-414 of SEQ ID NO:9 of at least 12, preferably at least 24, more preferably at least 36, and most preferably at least 96 contiguous nucleotides.

The subject nucleic acid sequences may consist solely of any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or fragments thereof. Alternatively, the subject nucleic acid sequences and fragments thereof may be joined to other components such as labels, peptides, agents that facilitate transport across cell membranes, hybridization-triggered cleavage agents or intercalating agents. The subject nucleic acid sequences and fragments thereof may also be joined to other nucleic acid sequences (i.e. they may comprise part of larger sequences) and are of synthetic/non-natural sequences and/or are isolated and/or are purified, i.e. unaccompanied by at least some of the material with which it is associated in its natural state. Preferably, the isolated nucleic acids constitute at least about 0.5%, and more preferably at least about 5% by weight of the total nucleic acid present in a given fraction, and are preferably recombinant, meaning that they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome.

Derivative nucleic acid sequences of p53 include sequences that hybridize to the nucleic acid sequence of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringency conditions such that the hybridizing derivative nucleic acid is related to the subject nucleic acid by a certain degree of sequence identity. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule. Stringency of hybridization refers to conditions under which nucleic acids are hybridizable. The degree of stringency can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. As used herein, the term "stringent hybridization conditions" are those normally used by one of skill in the art to establish at least about a 90% sequence identity between complementary pieces of DNA or DNA and RNA. "Moderately stringent hybridization conditions" are used to find derivatives having at least about a 70% sequence identity. Finally, "low-stringency hybridization conditions" are used to isolate derivative nucleic acid molecules that share at least about 50% sequence identity with the subject nucleic acid sequence.

The ultimate hybridization stringency reflects both the actual hybridization conditions as well as the washing conditions following the hybridization, and it is well known in the art how to vary the conditions to obtain the desired result. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). A preferred derivative nucleic acid is

capable of hybridizing to any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1 h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

Derivative nucleic acid sequences that have at least about 70% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 are capable of hybridizing to any one of SEQ ID NO:1, 3, 5, 7, 9, and 18 under moderately stringent conditions that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Other preferred derivative nucleic acid sequences are capable of hybridizing to any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 under low stringency conditions that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

As used herein, "percent (%) nucleic acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides in the candidate derivative nucleic acid sequence identical with the nucleotides in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410; <http://blast.wustl.edu/blast/README.html>; hereinafter referred to generally as "BLAST") with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database

against which the sequence of interest is being searched. A percent (%) nucleic acid sequence identity value is determined by the number of matching identical nucleotides divided by the sequence length for which the percent identity is being reported.

Derivative p53 nucleic acid sequences usually have at least 50% sequence identity, preferably at least 60%, 70%, or 80% sequence identity, more preferably at least 85% sequence identity, still more preferably at least 90% sequence identity, and most preferably at least 95% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or domain-encoding regions thereof.

In one preferred embodiment, the derivative nucleic acid encodes a polypeptide comprising a p53 amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, or 10, or a fragment or derivative thereof as described further below under the subheading "p53 proteins". A derivative p53 nucleic acid sequence, or fragment thereof, may comprise 100% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, but be a derivative thereof in the sense that it has one or more modifications at the base or sugar moiety, or phosphate backbone. Examples of modifications are well known in the art (Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such derivatives may be used to provide modified stability or any other desired property.

Another type of derivative of the subject nucleic acid sequences includes corresponding humanized sequences. A humanized nucleic acid sequence is one in which one or more codons has been substituted with a codon that is more commonly used in human genes. Preferably, a sufficient number of codons have been substituted such that a higher level expression is achieved in mammalian cells than what would otherwise be achieved without the substitutions. The following list shows, for each amino acid, the calculated codon frequency (number in parentheses) in human genes for 1000 codons (Wada *et al.*, Nucleic Acids Research (1990) 18(Suppl.):2367-2411):

Human codon frequency per 1000 codons:

ARG: CGA (5.4), CGC (11.3), CGG (10.4), CGU (4.7), AGA (9.9), AGG (11.1)
 LEU: CUA (6.2), CUC (19.9), CUG (42.5), CUU (10.7), UUA (5.3), UUG (11.0)
 SER: UCA (9.3), UCC (17.7), UCG (4.2), UCU (13.2), AGC (18.7), AGU (9.4)
 THR: ACA (14.4), ACC (23.0), ACG (6.7), ACU (12.7)
 PRO: CCA (14.6), CCC (20.0), CCG (6.6), CCU (15.5)
 ALA: GCA (14.0), GCC (29.1), GCG (7.2), GCU (19.6)
 GLY: GGA (17.1), GGC (25.4), GGG (17.3), GGU (11.2)
 VAL: GUA (5.9), GUC (16.3), GUG (30.9), GUU (10.4)
 LYS: AAA (22.2), AAG (34.9)
 ASN: AAC (22.6), AAU (16.6)
 GLN: CAA (11.1), CAG (33.6)

HIS: CAC (14.2), CAU (9.3)
 GLU: GAA (26.8), GAG (41.4)
 ASP: GAC (29.0), GAU (21.7)
 TYR: UAC (18.8), UAU (12.5)
 5 CYS: UGC (14.5), UGU (9.9)
 PHE: UUU (22.6), UUC (15.8)
 ILE: AUA (5.8), AUC (24.3), AUU (14.9)
 MET: AUG (22.3)
 TRP: UGG (13.8)
 10 TER: UAA (0.7), AUG (0.5), UGA (1.2)

Thus, a p53 nucleic acid sequence in which the glutamic acid codon, GAA has been replaced with the codon GAG, which is more commonly used in human genes, is an example of a humanized p53 nucleic acid sequence. A detailed discussion of the humanization of nucleic acid sequences is provided in U.S. Pat. No. 5,874,304 to Zolotukhin *et al.* Similarly, other nucleic acid derivatives can be generated with codon usage optimized for expression in other organisms, such as yeasts, bacteria, and plants, where it is desired to engineer the expression of p53 proteins by using specific codons chosen according to the preferred codons used in highly expressed genes in each organism.

20 More specific embodiments of preferred p53 proteins, fragments, and derivatives are discussed further below in connection under the subheading "p53 proteins".

Nucleic acid encoding the amino acid sequence of any of SEQ ID NOs:2, 4, 6, 8, and 10, or fragment or derivative thereof, may be obtained from an appropriate cDNA library prepared from any eukaryotic species that encodes p53 proteins such as vertebrates, preferably mammalian (*e.g.* primate, porcine, bovine, feline, equine, and canine species, *etc.*) and invertebrates, such as arthropods, particularly insects species (preferably *Drosophila*, *Tribolium*, *Leptinotarsa*, and *Heliothis*), *acarids*, *crustacea*, *molluscs*, *nematodes*, and other worms. An expression library can be constructed using known methods. For example, mRNA can be isolated to make cDNA which is ligated into a suitable expression vector for expression in a host cell into which it is introduced. Various screening assays can then be used to select for the gene or gene product (*e.g.* oligonucleotides of at least about 20 to 80 bases designed to identify the gene of interest, or labeled antibodies that specifically bind to the gene product). The gene and/or gene product can then be recovered from the host cell using known techniques.

35 Polymerase chain reaction (PCR) can also be used to isolate nucleic acids of the p53 genes where oligonucleotide primers representing fragmentary sequences of interest amplify RNA or DNA sequences from a source such as a genomic or cDNA library (as

described by Sambrook *et al.*, *supra*). Additionally, degenerate primers for amplifying homologs from any species of interest may be used. Once a PCR product of appropriate size and sequence is obtained, it may be cloned and sequenced by standard techniques, and utilized as a probe to isolate a complete cDNA or genomic clone.

5 Fragmentary sequences of p53 nucleic acids and derivatives may be synthesized by known methods. For example, oligonucleotides may be synthesized using an automated DNA synthesizer available from commercial suppliers (*e.g.* Biosearch, Novato, CA; Perkin-Elmer Applied Biosystems, Foster City, CA). Antisense RNA sequences can be produced intracellularly by transcription from an exogenous sequence, *e.g.* from vectors that contain
10 antisense p53 nucleic acid sequences. Newly generated sequences may be identified and isolated using standard methods.

 An isolated p53 nucleic acid sequence can be inserted into any appropriate cloning vector, for example bacteriophages such as lambda derivatives, or plasmids such as PBR322, pUC plasmid derivatives and the Bluescript vector (Stratagene, San Diego, CA).
15 Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, *etc.*, or into a transgenic animal such as a fly. The transformed cells can be cultured to generate large quantities of the p53 nucleic acid. Suitable methods for isolating and producing the subject nucleic acid sequences are well-known in the art (Sambrook *et al.*, *supra*; DNA Cloning: A Practical Approach, Vol. 1, 2, 3, 4, (1995)
20 Glover, ed., MRL Press, Ltd., Oxford, U.K.).

 The nucleotide sequence encoding a p53 protein or fragment or derivative thereof, can be inserted into any appropriate expression vector for the transcription and translation of the inserted protein-coding sequence. Alternatively, the necessary transcriptional and translational signals can be supplied by the native p53 gene and/or its flanking regions. A
25 variety of host-vector systems may be utilized to express the protein-coding sequence such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. If expression in plants is desired, a variety of transformation constructs, vectors and
30 methods are known in the art (see U.S. Pat. No. 6,002,068 for review). Expression of a p53 protein may be controlled by a suitable promoter/enhancer element. In addition, a host cell strain may be selected which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired

To detect expression of the p53 gene product, the expression vector can comprise a promoter operably linked to a p53 gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the
5 expression of the p53 gene product based on the physical or functional properties of the p53 protein in *in vitro* assay systems (*e.g.* immunoassays or cell cycle assays). The p53 protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product as described above.

Once a recombinant that expresses the p53 gene sequence is identified, the gene
10 product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). The amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant and can thus be synthesized by standard chemical methods (Hunkapiller *et al.*, *Nature* (1984) 310:105-111). Alternatively, native
15 p53 proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification).

p33 and Rb Nucleic Acids

The invention also provides nucleic acid sequences for *Drosophila* p33 (SEQ ID
20 NO:19), and Rb (SEQ ID NO:21) tumor suppressors. Derivatives and fragments of these sequences can be prepared as described above for the p53 sequences. Preferred fragments and derivatives comprise the same number of contiguous nucleotides or same degrees of percent identity as described above for p53 nucleic acid sequences. The disclosure below regarding various uses of p53 tumor suppressor nucleic acids and proteins (*e.g.* transgenic
25 animals, tumor suppressor assays, *etc.*) also applies to the p33 and Rb tumor suppressor sequences disclosed herein.

p53 Proteins

The CLUSTALW program (Thompson, *et al.*, *Nucleic Acids Research* (1994)
30 22(22):4673-4680) was used to align the insect p53 proteins described herein with p53 proteins from human (Zakut-Houri *et al.*, *EMBO J.* (1985) 4:1251-1255; GenBank gi:129369), *Xenopus* (Sousi *et al.*, *Oncogene* (1987) 1:71-78; GenBank gi:129374), and squid (GenBank gi:1244762). The alignment generated is shown in Figure 1 and reveals a number of features in the insect p53 proteins that are characteristic of the previously-

identified p53 proteins. With respect to general areas of structural similarity, the DMp53, CPBp53, and TRIB-Ap53 proteins can be roughly divided into three regions: a central region which exhibits a high degree of sequence homology with other known p53 family proteins and which roughly corresponds to the DNA binding domain of this protein family (Cho *et al.*, Science (1994) 265:346-355), and flanking N-terminal and C-terminal regions which exhibit significantly less homology but which correspond in overall size to other p53 family proteins. The fragmentary polypeptide sequences encoded by the TRIB-Bp53 and HELIOp53 cDNAs are shown by the multiple sequence alignment to be derived from the central region – the conserved DNA-binding domain. Significantly, the protein sequence alignment allowed the assignment of the domains in the DMp53, CPBp53, and TRIB-A p53 proteins listed in Table 1 above, based on sequence homology with previously characterized domains of human p53 (Soussi and May, J. Mol Biol (1996) 260:623-637; Levine, *supra*; Prives, Cell (1998) 95:5-8).

Importantly, the most conserved central regions of the DMp53, CPBp53, and TRIB-A p53 proteins correspond almost precisely to the known functional boundaries of the DNA binding domain of human p53, indicating that these proteins are likely to exhibit similar DNA binding properties to those of human p53. A detailed examination of the conserved residues in this domain further emphasizes the likely structural and functional similarities between human p53 and the insect p53 proteins. First, residues of the human p53 known to be involved in direct DNA contacts (K120, S241, R248, R273, C277, and R280) correspond to identical or similar residues in the DMp53 protein (K113, S230, R234, K259, C263, and R266), and identical residues in the CPBp53 protein (K92, S216, R224, R249, C253, and R256), and the TRIB-Ap53 protein (K88, S213, R220, R245, C249, and R252). Also, with regard to the overall folding of this domain, it was notable that four key residues that coordinate the zinc ligand in the DNA binding domain of human p53 (C176, H179, C238, and C242) are precisely conserved in the DMp53 protein (C156, H159, C227, and C231), the CPBp53 protein (C147, H150, C213, and C217), and the TRIB-A p53 protein (C144, H147, C210, C214). Furthermore, it was striking that the mutational hot spots in human p53 most frequently altered in cancer (R175, G245, R248, R249, R273, and R282), are either identical or conserved amino acid residues in the corresponding positions of the DMp53 protein (R155, G233, R234, K235, K259, and R268), the CPBp53 protein (R146, G221, R224, R225, R249, and K258), and the TRIB-Ap53 protein (R143, G217, R220, R221, R245, and K254).

Interestingly, the insect p53s also have distinct differences from the Human, *Xenopus*, and squid p53s. Specifically, insect p53s contain a unique amino acid sequence within the DNA recognition domain that has the following sequence: (R or K)(I or V)C(S or T)CPKRD. Specifically, amino acid residues 259 to 267 of DMp53 have the sequence: KICTCPKRD; residues 249 to 257 of CPBp53 have the sequence: RICSCPKRD; and residues 245-253 of TRIB-Ap53 have the sequence: RVCSCPKRD. This is in distinct contrast to the Human, *Xenopus*, and squid p53s which have the following corresponding sequence: R(I or V)CACPGRD.

Another region of insect p53s that distinctly differs from previously identified p53s lies in the zinc coordination region of the DNA binding domain. The following sequence is conserved within the insect p53s: FXC(K or Q)NSC (where X = any amino acid). Specifically, residues 225-231 of DMp53 have the sequence: FVCQNSC; residues 211-217 of CPBp53 and residues 208-214 of TRIB-Ap53 have the sequence FVCKNSC; and the corresponding residues in Helio-p53, as shown in Figure 1, have the sequence: FSCKNSC. In contrast, the corresponding sequence in Human and *Xenopus* p53 is YMCNSSC, and in squid it is FMCLGSC.

The high degree of structural homology in the presumptive DNA binding domain of the insect p53 proteins has important implications for engineering derivative (*e.g.* mutant) forms of these p53 genes for tests of function *in vitro* and *in vivo*, and for genetic dissection or manipulation of the p53 pathway in transgenic insects or insect cell lines. Dominant negative forms of human p53 have been generated by creating altered proteins which have a defective DNA binding domain, but which retain a functional oligomerization domain (Brachman *et al.*, Proc Natl Acad Sci USA (1996) 93:4091-4095). Such dominant negative mutant forms are extremely useful for determining the effects of loss-of-function of p53 in assays of interest. Thus, mutations in highly conserved positions within the DNA binding domain of the insect p53 proteins, which correspond to residues known to be important for the structure and function of human p53 (such as R175H, H179N, and R280T of human p53), are likely to result in dominant negative forms of insect p53 proteins. For example, specific mutations in the DMp53 protein to create dominant negative mutant forms of the protein include R155H, H159N, and R266T and for the TRIB-A p53 protein include R143H, H147N, and R252T.

Although other domains of the insect p53 proteins, aside from the DNA binding domain, exhibit significantly less homology compared to the known p53 family proteins, the sequence alignment provides important information about their structure and potential

function. Notably, just as in the human p53 protein, the C-terminal 20-25 amino acids of the protein comprise a putative region that extends beyond the oligomerization domain, suggesting an analogous function for this region of the insect p53 proteins in regulating activity of the protein. Since deletion of the C-terminal regulatory domain in human p53
5 has been shown to generate constitutively activated forms of the protein (Hupp and Lane, Curr. Biol. (1994) 4:865-875), it is expected that removal of most or all of the corresponding regulatory domain from the insect p53 proteins will generate an activated protein form. Thus preferred truncated forms of the insect p53 proteins lack at least 10 C-terminal amino acids, more preferably at least 15 amino acids, and most preferably at least
10 20 C-terminal amino acids. For example, a preferred truncated version of DMp53 comprises amino acid residues 1-376, more preferably residues 1-371, and most preferably residues 1-366 of SEQ ID NO:2. Such constitutively activated mutant forms of the protein are very useful for tests of protein function using *in vivo* and *in vitro* assays, as well as for genetic analysis.

15 The oligomerization domain of the insect p53 proteins exhibit very limited skeletal sequence homology with other p53 family proteins, although the length of this region is similar to that of other p53 family proteins. The extent of sequence divergence in this region of the insect proteins raises the possibility that the insect p53 protein may be unable to form hetero-oligomers with p53 proteins from vertebrates or squid. And, although the
20 linker domain located between the DNA binding and oligomerization domains also exhibits relatively little sequence conservation, this region of any of the DMp53, CPBp53, and TRIB-A p53 proteins contains predicted nuclear localization signals similar to those identified in human p53 (Shaulsky *et al.*, Mol Cell Biol (1990) 10:6565-6577).

The activation domain at the N-terminus of the insect p53 proteins also exhibits
25 little sequence identity with other p53 family proteins, although the size of this region is roughly the same as that of human p53. Nonetheless, an important feature of this domain is the relative concentration of acidic residues in the insect p53 proteins. Consequently, it is likely that this N-terminal domain of any of the DMp53, CPBp53, and TRIB-Ap53 proteins will similarly exert the functional activity of a transcriptional activation domain to that of
30 the human p53 domain (Thut *et al.*, Science (1995) 267:100-104). Interestingly, the DMp53, CPBp53 and TRIB-A p53 proteins do not appear to possess a highly conserved sequence motif, FxxLWxxL, found at the N-terminus of vertebrate and squid p53 family proteins. In the human p53 gene, these conserved residues in this motif participate in a

specific interaction between human p53 proteins and mdm2 (Kussie *et al.*, Science (1996) 274:948-953).

It is important to note that, although there is no sequence similarity between the insect p53s and other p53 family members in the C- and N-termini, these regions of p53 contain secondary structure characteristic of p53-related proteins. For example, the human p53 binds DNA as a homo-tetramer and self-association is mediated by a β -sheet and amphipathic α -helix located in the C-terminus of the protein. A similar β -sheet-turn- α -helix is predicted in the C-terminus of DMp53. Further, the N-terminus of the human p53 is a region that includes a transactivation domain and residues critical for binding to the mdm-2 protein. The N-terminus of the DMp53 also include acidic amino acids and likely functions as a transactivation domain.

p53 proteins of the invention comprise or consist of an amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments or derivatives thereof. Compositions comprising these proteins may consist essentially of the p53 protein, fragments, or derivatives, or may comprise additional components (*e.g.* pharmaceutically acceptable carriers or excipients, culture media, *etc.*). p53 protein derivatives typically share a certain degree of sequence identity or sequence similarity with any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments thereof. As used herein, "percent (%) amino acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of amino acids in the candidate derivative amino acid sequence identical with the amino acid in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by BLAST (Altschul *et al.*, *supra*) using the same parameters discussed above for derivative nucleic acid sequences. A % amino acid sequence identity value is determined by the number of matching identical amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation. A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine;

interchangeable basic amino acids arginine, lysine and histidine; interchangeable acidic amino acids aspartic acid and glutamic acid; and interchangeable small amino acids alanine, serine, cystine, threonine, and glycine.

In one preferred embodiment, a p53 protein derivative shares at least 50% sequence identity or similarity, preferably at least 60%, 70%, or 80% sequence identity or similarity, more preferably at least 85% sequence similarity or identity, still more preferably at least 90% sequence similarity or identity, and most preferably at least 95% sequence identity or similarity with a contiguous stretch of at least 10 amino acids, preferably at least 25 amino acids, more preferably at least 40 amino acids, still more preferably at least 50 amino acids, more preferably at least 100 amino acids, and in some cases, the entire length of any one of SEQ ID NOs: 2, 4, 6, 8, or 10. Further preferred derivatives share these % sequence identities with the domains of SEQ ID NOs 2, 4 and 6 listed in Table I above. Additional preferred derivatives comprise a sequence that shares 100% similarity with any contiguous stretch of at least 10 amino acids, preferably at least 12, more preferably at least 15, and most preferably at least 20 amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof. Further preferred fragments comprise at least 7 contiguous amino acids, preferably at least 9, more preferably at least 12, and most preferably at least 17 contiguous amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof.

Other preferred p53 polypeptides, fragments or derivatives consist of or comprise a sequence selected from the group consisting of RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, and KVCTCPGRD (i.e. sequences of the formula: (R or K)(I or V)C(S or T)CPGRD). Additional preferred p53 polypeptides, fragments or derivatives, consist of or comprise a sequence selected from the group consisting of FXCKNSC and FXCQNSC, where X = any amino acid.

The fragment or derivative of any of the p53 proteins is preferably "functionally active" meaning that the p53 protein derivative or fragment exhibits one or more functional activities associated with a full-length, wild-type p53 protein comprising the amino acid sequence of any of SEQ ID NOs: 2, 4, 6, 8, or 10. As one example, a fragment or derivative may have antigenicity such that it can be used in immunoassays, for immunization, for inhibition of p53 activity, *etc.*, as discussed further below regarding generation of antibodies to p53 proteins. Preferably, a functionally active p53 fragment or derivative is one that displays one or more biological activities associated with p53 proteins such as regulation of

the cell cycle, or transcription control. The functional activity of p53 proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey). Example 12 below describes a variety of suitable assays for assessing p53
5 function.

P 53 derivatives can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned p53 gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) (Wells *et al.*, Philos. Trans. R. Soc. London SerA (1986) 317:415),
10 followed by further enzymatic modification if desired, isolated, and ligated *in vitro*, and expressed to produce the desired derivative. Alternatively, a p53 gene can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. A variety of
15 mutagenesis techniques are known in the art such as chemical mutagenesis, *in vitro* site-directed mutagenesis (Carter *et al.*, Nucl. Acids Res. (1986) 13:4331), use of TAB[®] linkers (available from Pharmacia and Upjohn, Kalamazoo, MI), *etc.*

At the protein level, manipulations include post translational modification, *e.g.* glycosylation, acetylation, phosphorylation, amidation, derivatization by known
20 protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications may be carried out by known technique (*e.g.* specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*). Derivative proteins can also be chemically
25 synthesized by use of a peptide synthesizer, for example to introduce nonclassical amino acids or chemical amino acid analogs as substitutions or additions into the p53 protein sequence.

Chimeric or fusion proteins can be made comprising a p53 protein or fragment thereof (preferably comprising one or more structural or functional domains of the p53
30 protein) joined at its N- or C-terminus via a peptide bond to an amino acid sequence of a different protein. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other in the proper coding frame using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer.

p53 and Rb Proteins

The invention also provides amino acid sequences for *Drosophila* p53 (SEQ ID NO:20), and Rb (SEQ ID NO:22) tumor suppressors. Derivatives and fragments of these sequences can be prepared as described above for the p53 protein sequences. Preferred fragments and derivatives comprise the same number of contiguous amino acids or same degrees of percent identity or similarity as described above for p53 amino acid sequences.

p53 Gene Regulatory Elements

p53 gene regulatory DNA elements, such as enhancers or promoters that reside within the 5' UTRs of SEQ ID NOs 1, 3, and 5, as shown in Table I above, or within nucleotides 1-1225 of SEQ ID NO:18, can be used to identify tissues, cells, genes and factors that specifically control p53 protein production. Preferably at least 20, more preferably at least 25, and most preferably at least 50 contiguous nucleotides within the 5' UTRs are used. Analyzing components that are specific to p53 protein function can lead to an understanding of how to manipulate these regulatory processes, for either pesticide or therapeutic applications, as well as an understanding of how to diagnose dysfunction in these processes.

Gene fusions with the p53 regulatory elements can be made. For compact genes that have relatively few and small intervening sequences, such as those described herein for *Drosophila*, it is typically the case that the regulatory elements that control spatial and temporal expression patterns are found in the DNA immediately upstream of the coding region, extending to the nearest neighboring gene. Regulatory regions can be used to construct gene fusions where the regulatory DNAs are operably fused to a coding region for a reporter protein whose expression is easily detected, and these constructs are introduced as transgenes into the animal of choice. An entire regulatory DNA region can be used, or the regulatory region can be divided into smaller segments to identify sub-elements that might be specific for controlling expression a given cell type or stage of development. One suitable method to decipher regions containing regulatory sequences is by an *in vitro* CAT assay (Mercer, Crit. Rev. Euk. Gene Exp. (1992) 2:251-263; Sambrook *et al.*, *supra*; and Gorman *et al.*, Mol. Cell. Biol. (1992) 2:1044-1051). Additional reporter proteins that can be used for construction of these gene fusions include *E. coli* beta-galactosidase and green fluorescent protein (GFP). These can be detected readily *in situ*, and thus are useful for histological studies and can be used to sort cells that express p53 proteins (O'Kane and

Gehring PNAS (1987) 84(24):9123-9127; Chalfie *et al.*, Science (1994) 263:802-805; and Cumberledge and Krasnow (1994) Methods in Cell Biology 44:143-159). Recombinase proteins, such as FLP or cre, can be used in controlling gene expression through site-specific recombination (Golic and Lindquist (1989) Cell 59(3):499-509; White *et al.*, Science (1996) 271:805-807). Toxic proteins such as the reaper and hid cell death proteins, are useful to specifically ablate cells that normally express p53 proteins in order to assess the physiological function of the cells (Kingston. In Current Protocols in Molecular Biology (1998) Ausubel *et al.*, John Wiley & Sons, Inc. sections 12.0.3-12.10) or any other protein where it is desired to examine the function this particular protein specifically in cells that synthesize p53 proteins.

Alternatively, a binary reporter system can be used, similar to that described further below, where the p53 regulatory element is operably fused to the coding region of an exogenous transcriptional activator protein, such as the GAL4 or tTA activators described below, to create a p53 regulatory element "driver gene". For the other half of the binary system the exogenous activator controls a separate "target gene" containing a coding region of a reporter protein operably fused to a cognate regulatory element for the exogenous activator protein, such as UAS_G or a tTA-response element, respectively. An advantage of a binary system is that a single driver gene construct can be used to activate transcription from preconstructed target genes encoding different reporter proteins, each with its own uses as delineated above.

p53 regulatory element-reporter gene fusions are also useful for tests of genetic interactions, where the objective is to identify those genes that have a specific role in controlling the expression of p53 genes, or promoting the growth and differentiation of the tissues that expresses the p53 protein. p53 gene regulatory DNA elements are also useful in protein-DNA binding assays to identify gene regulatory proteins that control the expression of p53 genes. The gene regulatory proteins can be detected using a variety of methods that probe specific protein-DNA interactions well known to those skilled in the art (Kingston, *supra*) including *in vivo* footprinting assays based on protection of DNA sequences from chemical and enzymatic modification within living or permeabilized cells; and *in vitro* footprinting assays based on protection of DNA sequences from chemical or enzymatic modification using protein extracts, nitrocellulose filter-binding assays and gel electrophoresis mobility shift assays using radioactively labeled regulatory DNA elements mixed with protein extracts. Candidate p53 gene regulatory proteins can be purified using a combination of conventional and DNA-affinity purification techniques. Molecular cloning

strategies can also be used to identify proteins that specifically bind p53 gene regulatory DNA elements. For example, a *Drosophila* cDNA library in an expression vector, can be screened for cDNAs that encode p53 gene regulatory element DNA-binding activity. Similarly, the yeast "one-hybrid" system can be used (Li and Herskowitz, *Science* (1993) 262:1870-1874; Luo *et al.*, *Biotechniques* (1996) 20(4):564-568; Vidal *et al.*, *PNAS* (1996) 93(19):10315-10320).

Assays for tumor suppressor genes

The p53 tumor suppressor gene encodes a transcription factor implicated in regulation of cell proliferation, control of the cell cycle, and induction of apoptosis. Various experimental methods may be used to assess the role of the insect p53 genes in each of these areas.

Transcription activity assays

Due to its acidic region, wild type p53 binds both specifically and non-specifically to DNA in order to mediate its function (Zambetti and Levine, *supra*). Transcriptional regulation by the p53 protein or its fragments may be examined by any method known in the art. An electrophoretic mobility shift assay can be used to characterize DNA sequences to which p53 binds, and thus can assist in the identification of genes regulated by p53. Briefly, cells are grown and transfected with various amounts of wild type or mutated transcription factor of interest (in this case, p53), harvested 48 hr after transfection, and lysed to prepare nuclear extracts. Preparations of *Drosophila* nuclear extracts for use in mobility shift assays may be done as described in Dignam *et al.*, *Nucleic Acids Res.* (1983) 11:1475-1489. Additionally, complementary, single-stranded oligonucleotides corresponding to target sequences for binding are synthesized and self-annealed to a final concentration of 10-15 ng/ μ l. Double stranded DNA is verified by gel electrophoretic analysis (*e.g.*, on a 7% polyacrylamide gel, by methods known in the art), and end-labeled with 20 μ Ci [32 P] γ -dATP. The nuclear extracts are mixed with the double stranded target sequences under conditions conducive for binding and the results are analyzed by polyacrylamide gel electrophoresis.

Another suitable method to determine DNA sequences to which p53 binds is by DNA footprinting (Schmitz *et al.*, *Nucleic Acids Research* (1978) 5:3157-3170).

Apoptosis assays

A variety of methods may be used to examine apoptosis. One method is the terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling

(TUNEL) assay which measures the nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, Nature (1994) 371:346-347; White *et al.*, Science (1994) 264:677-683).

Additionally, commercial kits can be used for detection of apoptosis (ApoAlert® available from Clontech (Palo Alto, CA)).

5 Apoptosis may also be assayed by a variety of staining methods. Acridine orange can be used to detect apoptosis in cultured cells (Lucas *et al.*, Blood (1998) 15:4730-41) and in intact *Drosophila* tissues, which can also be stained with Nile Blue (Abrams *et al.*, Development (1993) 117:29-43). Another assay that can be used to detect DNA laddering employs ethidium bromide staining and electrophoresis of DNA on an agarose gel (Civielli
10 *et al.*, Int. J. Cancer (1995) 27:673-679; Young, J. Biol. Chem. (1998) 273:25198-25202).

Proliferation and cell cycle assays

Proliferating cells may be identified by bromodeoxyuridine (BRDU) incorporation into cells undergoing DNA synthesis and detection by an anti-BRDU antibody (Hoshino *et al.*, Int. J. Cancer (1986) 38:369; Campana *et al.*, J. Immunol. Meth. (1988) 107:79). This
15 assay can be used to reproducibly identify S-phase cells in *Drosophila* embryos (Edgar and O'Farrell, Cell (1990) 62:469-480) and imaginal discs (Secombe *et al.*, Genetics (1998) 149:1867-1882). S-phase DNA syntheses can also be quantified by measuring [³H]-thymidine incorporation using a scintillation counter (Chen, Oncogene (1996) 13:1395-403; Jeoung, J. Biol. Chem. (1995) 270:18367-73). Cell proliferation may be measured by
20 counting samples of a cell population over time, for example using a hemacytometer and Trypan-blue staining.

The DNA content and/or mitotic index of the cells may be measured based on the DNA ploidy value of the cell using a variety of methods known in the art such as a propidium iodide assay (Turner *et al.*, Prostate (1998) 34:175-81) or Feulgen staining using
25 a computerized microdensitometry staining system (Bacus, Am. J. Pathol. (1989) 135:783-92).

The effect of p53 overexpression or loss-of-function on *Drosophila* cell proliferation can be assayed *in vivo* using an assay in which clones of cells with altered gene expression are generated in the developing wing disc of *Drosophila* (Neufeld *et al.*, Cell (1998)
30 93:1183-93). The clones coexpress GFP, which allows the size and DNA content of the mutant and wild-type cells from dissociated discs to be compared by FACS analysis.

Tumor formation and transformation assays

A variety of *in vivo* and *in vitro* tumor formation assays are known in the art that can be used to assay p53 function. Such assays can be used to detect foci formation (Beenken,

J. Surg. Res. (1992) 52:401-5), *in vitro* transformation (Ginsberg, Oncogene. (1991) 6:669-72), tumor formation in nude mice (Endlich, Int. J. Radiat. Biol. (1993) 64:715-26), tumor formation in *Drosophila* (Tao *et al.*, Nat. Genet. (1999) 21:177-181), and anchorage-independent growth in soft agar (Endlich, *supra*). Loss of indicia of
5 differentiation may be indicate transformation, including loss of differentiation markers, cell rounding, loss of adhesion, loss of polarity, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, and expression of fetal antigens.

10 **Generation and Genetic Analysis of Animals and Cell Lines with Altered Expression of p53 Gene**

Both genetically modified animal models (i.e. *in vivo* models), such as *C. elegans* and *Drosophila*, and *in vitro* models such as genetically engineered cell lines expressing or mis-expressing p53 genes, are useful for the functional analysis of these proteins. Model
15 systems that display detectable phenotypes, can be used for the identification and characterization of p53 genes or other genes of interest and/or phenotypes associated with the mutation or mis-expression of p53. The term "mis-expression" as used herein encompasses mis-expression due to gene mutations. Thus, a mis-expressed p53 protein may be one having an amino acid sequence that differs from wild-type (i.e. it is a derivative
20 of the normal protein). A mis-expressed p53 protein may also be one in which one or more N- or C- terminal amino acids have been deleted, and thus is a "fragment" of the normal protein. As used herein, "mis-expression" also includes ectopic expression (*e.g.* by altering the normal spatial or temporal expression), over-expression (*e.g.* by multiple gene copies), underexpression, non-expression (*e.g.* by gene knockout or blocking expression that would
25 otherwise normally occur), and further, expression in ectopic tissues.

The *in vivo* and *in vitro* models may be genetically engineered or modified so that they 1) have deletions and/or insertions of a p53 genes, 2) harbor interfering RNA sequences derived from a p53 gene, 3) have had an endogenous p53 gene mutated (*e.g.* contain deletions, insertions, rearrangements, or point mutations in the p53 gene), and/or 4)
30 contain transgenes for mis-expression of wild-type or mutant forms of a p53 gene. Such genetically modified *in vivo* and *in vitro* models are useful for identification of genes and proteins that are involved in the synthesis, activation, control, *etc.* of p53, and also downstream effectors of p53 function, genes regulated by p53, *etc.* The model systems can be used for testing potential pharmaceutical and pesticidal compounds that interact with

p53, for example by administering the compound to the model system using any suitable method (*e.g.* direct contact, ingestion, injection, *etc.*) and observing any changes in phenotype, for example defective movement, lethality, *etc.* Various genetic engineering and expression modification methods which can be used are well-known in the art, including chemical mutagenesis, transposon mutagenesis, antisense RNAi, dsRNAi, and transgene-mediated mis-expression.

Generating Loss-of-function Mutations by Mutagenesis

Loss-of-function mutations in an insect p53 gene can be generated by any of several mutagenesis methods known in the art (Ashburner. In *Drosophila melanogaster*: A Laboratory Manual (1989), Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press: pp. 299-418; Fly pushing: The Theory and Practice of *Drosophila melanogaster* Genetics (1997) Cold Spring Harbor Press, Plainview, NY, hereinafter "Fly Pushing"). Techniques for producing mutations in a gene or genome include use of radiation (*e.g.*, X-ray, UV, or gamma ray); chemicals (*e.g.*, EMS, MMS, ENU, formaldehyde, *etc.*); and insertional mutagenesis by mobile elements including dysgenesis induced by transposon insertions, or transposon-mediated deletions, for example, male recombination, as described below. Other methods of altering expression of genes include use of transposons (*e.g.*, P element, EP-type "overexpression trap" element, mariner element, *piggyBac* transposon, hermes, minos, sleeping beauty, *etc.*) to misexpress genes; antisense; double-stranded RNA interference; peptide and RNA aptamers; directed deletions; homologous recombination; dominant negative alleles; and intrabodies.

Transposon insertions lying adjacent to a p53 gene can be used to generate deletions of flanking genomic DNA, which if induced in the germline, are stably propagated in subsequent generations. The utility of this technique in generating deletions has been demonstrated and is well-known in the art. One version of the technique using collections of P element transposon induced recessive lethal mutations (P lethals) is particularly suitable for rapid identification of novel, essential genes in *Drosophila* (Cooley *et al.*, Science (1988) 239:1121-1128; Spralding *et al.*, PNAS (1995) 92:10824-10830). Since the sequence of the P elements are known, the genomic sequence flanking each transposon insert is determined either by plasmid rescue (Hamilton *et al.*, PNAS (1991) 88:2731-2735) or by inverse polymerase chain reaction (Rehm, <http://www.fruitfly.org/methods/>). A more recent version of the transposon insertion technique in male *Drosophila* using P elements is known as P-mediated male recombination (Preston and Engels, Genetics (1996) 144:1611-1638).

Generating Loss-of-function Phenotypes Using RNA-based Methods

p53 genes may be identified and/or characterized by generating loss-of-function phenotypes in animals of interest through RNA-based methods, such as antisense RNA (Schubiger and Edgar, *Methods in Cell Biology* (1994) 44:697-713). One form of the antisense RNA method involves the injection of embryos with an antisense RNA that is partially homologous to the gene of interest (in this case the p53 gene). Another form of the antisense RNA method involves expression of an antisense RNA partially homologous to the gene of interest by operably joining a portion of the gene of interest in the antisense orientation to a powerful promoter that can drive the expression of large quantities of antisense RNA, either generally throughout the animal or in specific tissues. Antisense RNA-generated loss-of-function phenotypes have been reported previously for several *Drosophila* genes including cactus, pecanex, and Krüppel (LaBonne *et al.*, *Dev. Biol.* (1989) 136(1):1-16; Schuh and Jackle, *Genome* (1989) 31(1):422-425; Geisler *et al.*, *Cell* (1992) 71(4):613-621).

Loss-of-function phenotypes can also be generated by cosuppression methods (Bingham, *Cell* (1997) 90(3):385-387; Smyth, *Curr. Biol.* (1997) 7(12):793-795; Que and Jorgensen, *Dev. Genet.* (1998) 22(1):100-109). Cosuppression is a phenomenon of reduced gene expression produced by expression or injection of a sense strand RNA corresponding to a partial segment of the gene of interest. Cosuppression effects have been employed extensively in plants and *C. elegans* to generate loss-of-function phenotypes. Cosuppression in *Drosophila* has been shown, where reduced expression of the Adh gene was induced from a white-Adh transgene (Pal-Bhadra *et al.*, *Cell* (1997) 90(3):479-490).

Another method for generating loss-of-function phenotypes is by double-stranded RNA interference (dsRNAi). This method is based on the interfering properties of double-stranded RNA derived from the coding regions of gene, and has proven to be of great utility in genetic studies of *C. elegans* (Fire *et al.*, *Nature* (1998) 391:806-811), and can also be used to generate loss-of-function phenotypes in *Drosophila* (Kennerdell and Carthew, *Cell* (1998) 95:1017-1026; Misquitta and Patterson *PNAS* (1999) 96:1451-1456).

Complementary sense and antisense RNAs derived from a substantial portion of a gene of interest, such as p53 gene, are synthesized *in vitro*, annealed in an injection buffer, and introduced into animals by injection or other suitable methods such as by feeding, soaking the animals in a buffer containing the RNA, *etc.* Progeny of the dsRNA treated animals are then inspected for phenotypes of interest (PCT publication no. WO99/32619).

dsRNAi can also be achieved by causing simultaneous expression *in vivo* of both sense and antisense RNA from appropriately positioned promoters operably fused to p53 sequences. Alternatively, the living food of an animal can be engineered to express sense and antisense RNA, and then fed to the animal. For example, *C. elegans* can be fed engineered *E. coli*, *Drosophila* can be fed engineered baker's yeast, and insects such as *Leptinotarsa* and *Heliothis* and other plant-eating animals can be fed transgenic plants engineered to produce the dsRNA.

RNAi has also been successfully used in cultured *Drosophila* cells to inhibit expression of targeted proteins (Dixon lab, University of Michigan, <http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html>). Thus, cell lines in culture can be manipulated using RNAi both to perturb and study the function of p53 pathway components and to validate the efficacy of therapeutic or pesticidal strategies which involve the manipulation of this pathway. A suitable protocol is described in Example 13.

15 **Generating Loss-of-function Phenotypes Using Peptide and RNA Aptamers**

Another method for generating loss-of-function phenotypes is by the use of peptide aptamers, which are peptides or small polypeptides that act as dominant inhibitors of protein function. Peptide aptamers specifically bind to target proteins, blocking their function ability (Kolonin and Finley, PNAS (1998) 95:14266-14271). Due to the highly selective nature of peptide aptamers, they may be used not only to target a specific protein, but also to target specific functions of a given protein (*e.g.* transcription function). Further, peptide aptamers may be expressed in a controlled fashion by use of promoters which regulate expression in a temporal, spatial or inducible manner. Peptide aptamers act dominantly; therefore, they can be used to analyze proteins for which loss-of-function mutants are not available.

Peptide aptamers that bind with high affinity and specificity to a target protein may be isolated by a variety of techniques known in the art. In one method, they are isolated from random peptide libraries by yeast two-hybrid screens (Xu *et al.*, PNAS (1997) 94:12473-12478). They can also be isolated from phage libraries (Hoogenboom *et al.*, Immunotechnology (1998) 4:1-20) or chemically generated peptides/libraries.

RNA aptamers are specific RNA ligands for proteins, that can specifically inhibit protein function of the gene (Good *et al.*, Gene Therapy (1997) 4:45-54; Ellington, *et al.*, Biotechnol. Annu. Rev. (1995) 1:185-214). *In vitro* selection methods can be used to identify RNA aptamers having a selected specificity (Bell *et al.*, J. Biol. Chem. (1998)

273:14309-14314). It has been demonstrated that RNA aptamers can inhibit protein function in *Drosophila* (Shi *et al.*, Proc. Natl. Acad. Sci USA (1999) 96:10033-10038). Accordingly, RNA aptamers can be used to decrease the expression of p53 protein or derivative thereof, or a protein that interacts with the p53 protein.

5 Transgenic animals can be generated to test peptide or RNA aptamers *in vivo* (Kolonin and Finley, *supra*). For example, transgenic *Drosophila* lines expressing the desired aptamers may be generated by P element mediated transformation (discussed below). The phenotypes of the progeny expressing the aptamers can then be characterized.

Generating Loss of Function Phenotypes Using Intrabodies

10 Intracellularly expressed antibodies, or intrabodies, are single-chain antibody molecules designed to specifically bind and inactivate target molecules inside cells. Intrabodies have been used in cell assays and in whole organisms such as *Drosophila* (Chen *et al.*, Hum. Gen. Ther. (1994) 5:595-601; Hassanzadeh *et al.*, Febs Lett. (1998) 16(1, 2):75-80 and 81-86). Inducible expression vectors can be constructed with intrabodies that
15 react specifically with p53 protein. These vectors can be introduced into model organisms and studied in the same manner as described above for aptamers.

Transgenesis

Typically, transgenic animals are created that contain gene fusions of the coding regions of the p53 gene (from either genomic DNA or cDNA) or genes engineered to
20 encode antisense RNAs, cosuppression RNAs, interfering dsRNA, RNA aptamers, peptide aptamers, or intrabodies operably joined to a specific promoter and transcriptional enhancer whose regulation has been well characterized, preferably heterologous promoters/enhancers (i.e. promoters/enhancers that are non-native to the p53 genes being expressed).

Methods are well known for incorporating exogenous nucleic acid sequences into
25 the genome of animals or cultured cells to create transgenic animals or recombinant cell lines. For invertebrate animal models, the most common methods involve the use of transposable elements. There are several suitable transposable elements that can be used to incorporate nucleic acid sequences into the genome of model organisms. Transposable elements are also particularly useful for inserting sequences into a gene of interest so that
30 the encoded protein is not properly expressed, creating a "knock-out" animal having a loss-of-function phenotype. Techniques are well-established for the use of P element in *Drosophila* (Rubin and Spradling, Science (1982) 218:348-53; U.S. Pat. No. 4,670,388). Additionally, transposable elements that function in a variety of species, have been

identified, such as PiggyBac (Thibault *et al.*, Insect Mol Biol (1999) 8(1):119-23), hobo, and hermes.

P elements, or marked P elements, are preferred for the isolation of loss-of-function mutations in *Drosophila* p53 genes because of the precise molecular mapping of these genes, depending on the availability and proximity of preexisting P element insertions for use as a localized transposon source (Hamilton and Zinn, Methods in Cell Biology (1994) 44:81-94; and Wolfner and Goldberg, Methods in Cell Biology (1994) 44:33-80). Typically, modified P elements are used which contain one or more elements that allow detection of animals containing the P element. Most often, marker genes are used that affect the eye color of *Drosophila*, such as derivatives of the *Drosophila white* or *rosy* genes (Rubin and Spradling, *supra*; and Klemenz *et al.*, Nucleic Acids Res. (1987) 15(10):3947-3959). However, in principle, any gene can be used as a marker that causes a reliable and easily scored phenotypic change in transgenic animals. Various other markers include bacterial plasmid sequences having selectable markers such as ampicillin resistance (Steller and Pirrotta, EMBO. J. (1985) 4:167-171); and *lacZ* sequences fused to a weak general promoter to detect the presence of enhancers with a developmental expression pattern of interest (Bellen *et al.*, Genes Dev. (1989) 3(9):1288-1300). Other examples of marked P elements useful for mutagenesis have been reported (Nucleic Acids Research (1998) 26:85-88; and <http://flybase.bio.indiana.edu>).

A preferred method of transposon mutagenesis in *Drosophila* employs the "local hopping" method (Tower *et al.* (Genetics (1993) 133:347-359). Each new P insertion line can be tested molecularly for transposition of the P element into the gene of interest (*e.g.* p53) by assays based on PCR. For each reaction, one PCR primer is used that is homologous to sequences contained within the P element and a second primer is homologous to the coding region or flanking regions of the gene of interest. Products of the PCR reactions are detected by agarose gel electrophoresis. The sizes of the resulting DNA fragments reveal the site of P element insertion relative to the gene of interest. Alternatively, Southern blotting and restriction mapping using DNA probes derived from genomic DNA or cDNAs of the gene of interest can be used to detect transposition events that rearrange the genomic DNA of the gene. P transposition events that map to the gene of interest can be assessed for phenotypic effects in heterozygous or homozygous mutant *Drosophila*.

In another embodiment, *Drosophila* lines carrying P insertions in the gene of interest, can be used to generate localized deletions using known methods (Kaiser,

Bioassays (1990) 12(6):297-301; Harnessing the power of *Drosophila* genetics, In *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology*, Goldstein and Fyrberg, Eds., Academic Press, Inc: San Diego, California). This is particularly useful if no P element transpositions are found that disrupt the gene of interest. Briefly, flies containing
 5 P elements inserted near the gene of interest are exposed to a further round of transposase to induce excision of the element. Progeny in which the transposon has excised are typically identified by loss of the eye color marker associated with the transposable element. The resulting progeny will include flies with either precise or imprecise excision of the P element, where the imprecise excision events often result in deletion of genomic DNA
 10 neighboring the site of P insertion. Such progeny are screened by molecular techniques to identify deletion events that remove genomic sequence from the gene of interest, and assessed for phenotypic effects in heterozygous and homozygous mutant *Drosophila*.

Recently a transgenesis system has been described that may have universal applicability in all eye-bearing animals and which has been proven effective in delivering
 15 transgenes to diverse insect species (Berghammer *et al.*, Nature (1999) 402:370-371). This system includes: an artificial promoter active in eye tissue of all animal species, preferably containing three Pax6 binding sites positioned upstream of a TATA box (3xP3; Sheng *et al.* Genes Devel. (1997) 11:1122-1131); a strong and visually detectable marker gene, such as GFP or other autofluorescent protein genes (Pasher *et al.*, Gene (1992) 111:229-233;
 20 U.S. Pat. No. 5,491,084); and promiscuous vectors capable of delivering transgenes to a broad range of animal species, for example transposon-based vectors derived from *Hermes*, *PiggyBac*, or *mariner*, or vectors based on pantropic VSV_G-pseudotyped retroviruses (Burns *et al.*, In Vitro Cell Dev Biol Anim (1996) 32:78-84; Jordan *et al.*, Insect Mol Biol (1998) 7: 215-222; US Pat. No. 5,670,345). Since the same transgenesis system can be
 25 used in a variety of phylogenetically diverse animals, comparative functional studies are greatly facilitated, which is especially helpful in evaluating new applications to pest management.

In addition to creating loss-of-function phenotypes, transposable elements can be used to incorporate p53, or fragments or derivatives thereof, as an additional gene into any
 30 region of an animal's genome resulting in mis-expression (including over-expression) of the gene. A preferred vector designed specifically for misexpression of genes in transgenic *Drosophila*, is derived from pGMR (Hay *et al.*, Development (1994) 120:2121-2129), is 9Kb long, and contains: an origin of replication for *E. coli*; an ampicillin resistance gene; P element transposon 3' and 5' ends to mobilize the inserted sequences; a White marker gene;

an expression unit comprising the TATA region of hsp70 enhancer and the 3'untranslated region of α -tubulin gene. The expression unit contains a first multiple cloning site (MCS) designed for insertion of an enhancer and a second MCS located 500 bases downstream, designed for the insertion of a gene of interest. As an alternative to transposable elements, homologous recombination or gene targeting techniques can be used to substitute a heterologous p53 gene or fragment or derivative for one or both copies of the animal's homologous gene. The transgene can be under the regulation of either an exogenous or an endogenous promoter element, and be inserted as either a minigene or a large genomic fragment. Gene function can be analyzed by ectopic expression, using, for example, *Drosophila* (Brand *et al.*, Methods in Cell Biology (1994) 44:635- 654).

Examples of well-characterized heterologous promoters that may be used to create transgenic *Drosophila* include heat shock promoters/enhancers such as the *hsp70* and *hsp83* genes. Eye tissue specific promoters/enhancers include *eyeless* (Mozer and Benzer, Development (1994) 120:1049-1058), *sevenless* (Bowtell *et al.*, PNAS (1991) 88(15):6853-6857), and *glass*-responsive promoters/enhancers (Quiring *et al.*, Science (1994) 265:785-789). Wing tissue specific enhancers/promoters can be derived from the *dpp* or *vestigal* genes (Staehling-Hampton *et al.*, Cell Growth Differ. (1994) 5(6):585-593; Kim *et al.*, Nature (1996) 382:133-138). Finally, where it is necessary to restrict the activity of dominant active or dominant negative transgenes to regions where p53 is normally active, it may be useful to use endogenous p53 promoters. The ectopic expression of DMp53 in *Drosophila* larval eye using *glass*-responsive enhancer elements is described in Example 12 below.

In *Drosophila*, binary control systems that employ exogenous DNA are useful when testing the mis-expression of genes in a wide variety of developmental stage-specific and tissue-specific patterns. Two examples of binary exogenous regulatory systems include the UAS/GAL4 system from yeast (Hay *et al.*, PNAS (1997) 94(10):5195-5200; Ellis *et al.*, Development (1993) 119(3):855-865), and the "Tet system" derived from *E. coli* (Bello *et al.*, Development (1998) 125:2193-2202). The UAS/GAL4 system is a well-established and powerful method of mis-expression which employs the UAS_G upstream regulatory sequence for control of promoters by the yeast GAL4 transcriptional activator protein (Brand and Perrimon, Development (1993) 118(2):401-15). In this approach, transgenic *Drosophila*, termed "target" lines, are generated where the gene of interest to be mis-expressed is operably fused to an appropriate promoter controlled by UAS_G. Other transgenic *Drosophila* strains, termed "driver" lines, are generated where the GAL4 coding

region is operably fused to promoters/enhancers that direct the expression of the GAL4 activator protein in specific tissues, such as the eye, wing, nervous system, gut, or musculature. The gene of interest is not expressed in the target lines for lack of a transcriptional activator to drive transcription from the promoter joined to the gene of interest. However, when the UAS-target line is crossed with a GAL4 driver line, mis-expression of the gene of interest is induced in resulting progeny in a specific pattern that is characteristic for that GAL4 line. The technical simplicity of this approach makes it possible to sample the effects of directed mis-expression of the gene of interest in a wide variety of tissues by generating one transgenic target line with the gene of interest, and crossing that target line with a panel of pre-existing driver lines.

In the "Tet" binary control system, transgenic *Drosophila* driver lines are generated where the coding region for a tetracycline-controlled transcriptional activator (tTA) is operably fused to promoters/enhancers that direct the expression of tTA in a tissue-specific and/or developmental stage-specific manner. The driver lines are crossed with transgenic *Drosophila* target lines where the coding region for the gene of interest to be mis-expressed is operably fused to a promoter that possesses a tTA-responsive regulatory element. When the resulting progeny are supplied with food supplemented with a sufficient amount of tetracycline, expression of the gene of interest is blocked. Expression of the gene of interest can be induced at will simply by removal of tetracycline from the food. Also, the level of expression of the gene of interest can be adjusted by varying the level of tetracycline in the food. Thus, the use of the Tet system as a binary control mechanism for mis-expression has the advantage of providing a means to control the amplitude and timing of mis-expression of the gene of interest, in addition to spatial control. Consequently, if a p53 gene has lethal or deleterious effects when mis-expressed at an early stage in development, such as the embryonic or larval stages, the function of the gene in the adult can still be assessed by adding tetracycline to the food during early stages of development and removing tetracycline later so as to induce mis-expression only at the adult stage.

Dominant negative mutations, by which the mutation causes a protein to interfere with the normal function of a wild-type copy of the protein, and which can result in loss-of-function or reduced-function phenotypes in the presence of a normal copy of the gene, can be made using known methods (Hershkowitz, Nature (1987) 329:219-222). In the case of active monomeric proteins, overexpression of an inactive form, achieved, for example, by linking the mutant gene to a highly active promoter, can cause competition for natural substrates or ligands sufficient to significantly reduce net activity of the normal protein.

Alternatively, changes to active site residues can be made to create a virtually irreversible association with a target.

Assays for Change in Gene Expression

5 Various expression analysis techniques may be used to identify genes which are differentially expressed between a cell line or an animal expressing a wild type p53 gene compared to another cell line or animal expressing a mutant p53 gene. Such expression profiling techniques include differential display, serial analysis of gene expression (SAGE), transcript profiling coupled to a gene database query, nucleic acid array technology,
10 subtractive hybridization, and proteome analysis (*e.g.* mass-spectrometry and two-dimensional protein gels). Nucleic acid array technology may be used to determine the genome-wide expression pattern in a normal animal for comparison with an animal having a mutation in the p53 gene. Gene expression profiling can also be used to identify other genes or proteins that may have a functional relation to p53. The genes are identified by
15 detecting changes in their expression levels following mutation, over-expression, under-expression, mis-expression or knock-out, of the p53 gene.

Phenotypes Associated With p53 Gene Mutations

After isolation of model animals carrying mutated or mis-expressed p53 genes or
20 inhibitory RNAs, animals are carefully examined for phenotypes of interest. For analysis of p53 genes that have been mutated, animal models that are both homozygous and heterozygous for the altered p53 gene are analyzed. Examples of specific phenotypes that may be investigated include lethality; sterility; feeding behavior, tumor formation, perturbations in neuromuscular function including alterations in motility, and alterations in
25 sensitivity to pharmaceuticals. Some phenotypes more specific to flies include alterations in: adult behavior such as, flight ability, walking, grooming, phototaxis, mating or egg-laying; alterations in the responses of sensory organs, changes in the morphology, size or number of adult tissues such as, eyes, wings, legs, bristles, antennae, gut, fat body, gonads, and musculature; larval tissues such as mouth parts, cuticles, internal tissues or imaginal
30 discs; or larval behavior such as feeding, molting, crawling, or puparian formation; or developmental defects in any germline or embryonic tissues.

Genomic sequences containing a p53 gene can be used to engineer an existing mutant insect line, using the transgenesis methods previously described, to determine whether the mutation is in the p53 gene. Briefly, germline transformants are crossed for

complementation testing to an existing or newly created panel of insect lines whose mutations have been mapped to the vicinity of the gene of interest (Fly Pushing, *supra*). If a mutant line is discovered to be rescued by the genomic fragment, as judged by complementation of the mutant phenotype, then the mutant line likely harbors a mutation in the p53 gene. This prediction can be further confirmed by sequencing the p53 gene from the mutant line to identify the lesion in the p53 gene.

Identification of Genes That Modify p53 Genes

The characterization of new phenotypes created by mutations or misexpression in p53 genes enables one to test for genetic interactions between p53 genes and other genes that may participate in the same, related, or interacting genetic or biochemical pathway(s). Individual genes can be used as starting points in large-scale genetic modifier screens as described in more detail below. Alternatively, RNAi methods can be used to simulate loss-of-function mutations in the genes being analyzed. It is of particular interest to investigate whether there are any interactions of p53 genes with other well-characterized genes, particularly genes involved in regulation of the cell cycle or apoptosis.

Genetic Modifier Screens

A genetic modifier screen using invertebrate model organisms is a particularly preferred method for identifying genes that interact with p53 genes, because large numbers of animals can be systematically screened making it more possible that interacting genes will be identified. In *Drosophila*, a screen of up to about 10,000 animals is considered to be a pilot-scale screen. Moderate-scale screens usually employ about 10,000 to about 50,000 flies, and large-scale screens employ greater than about 50,000 flies. In a genetic modifier screen, animals having a mutant phenotype due to a mutation in or misexpression of the p53 gene are further mutagenized, for example by chemical mutagenesis or transposon mutagenesis.

The procedures involved in typical *Drosophila* genetic modifier screens are well-known in the art (Wolfner and Goldberg, *Methods in Cell Biology* (1994) 44:33-80; and Karim *et al.*, *Genetics* (1996) 143:315-329). The procedures used differ depending upon the precise nature of the mutant allele being modified. If the mutant allele is genetically recessive, as is commonly the situation for a loss-of-function allele, then most typically males, or in some cases females, which carry one copy of the mutant allele are exposed to an effective mutagen, such as EMS, MMS, ENU, triethylamine, diepoxylkanes, ICR-170, formaldehyde, X-rays, gamma rays, or ultraviolet radiation. The mutagenized animals are

crossed to animals of the opposite sex that also carry the mutant allele to be modified. In the case where the mutant allele being modified is genetically dominant, as is commonly the situation for ectopically expressed genes, wild type males are mutagenized and crossed to females carrying the mutant allele to be modified.

5 The progeny of the mutagenized and crossed flies that exhibit either enhancement or suppression of the original phenotype are presumed to have mutations in other genes, called "modifier genes", that participate in the same phenotype-generating pathway. These progeny are immediately crossed to adults containing balancer chromosomes and used as founders of a stable genetic line. In addition, progeny of the founder adult are retested
10 under the original screening conditions to ensure stability and reproducibility of the phenotype. Additional secondary screens may be employed, as appropriate, to confirm the suitability of each new modifier mutant line for further analysis.

Standard techniques used for the mapping of modifiers that come from a genetic screen in *Drosophila* include meiotic mapping with visible or molecular genetic markers;
15 male-specific recombination mapping relative to P-element insertions; complementation analysis with deficiencies, duplications, and lethal P-element insertions; and cytological analysis of chromosomal aberrations (Fly Pushing, *supra*). Genes corresponding to modifier mutations that fail to complement a lethal P-element may be cloned by plasmid rescue of the genomic sequence surrounding that P-element. Alternatively, modifier genes
20 may be mapped by phenotype rescue and positional cloning (Sambrook *et al.*, *supra*).

Newly identified modifier mutations can be tested directly for interaction with other genes of interest known to be involved or implicated with p53 genes using methods described above. Also, the new modifier mutations can be tested for interactions with genes in other pathways that are not believed to be related to regulation of cell cycle or apoptosis.
25 New modifier mutations that exhibit specific genetic interactions with other genes implicated in cell cycle regulation or apoptosis, and not with genes in unrelated pathways, are of particular interest.

The modifier mutations may also be used to identify "complementation groups". Two modifier mutations are considered to fall within the same complementation group if
30 animals carrying both mutations in trans exhibit essentially the same phenotype as animals that are homozygous for each mutation individually and, generally are lethal when in trans to each other (Fly Pushing, *supra*). Generally, individual complementation groups defined in this way correspond to individual genes.

When p53 modifier genes are identified, homologous genes in other species can be isolated using procedures based on cross-hybridization with modifier gene DNA probes, PCR-based strategies with primer sequences derived from the modifier genes, and/or computer searches of sequence databases. For therapeutic applications related to the function of p53 genes, human and rodent homologs of the modifier genes are of particular interest.

Although the above-described *Drosophila* genetic modifier screens are quite powerful and sensitive, some genes that interact with p53 genes may be missed in this approach, particularly if there is functional redundancy of those genes. This is because the vast majority of the mutations generated in the standard mutagenesis methods will be loss-of-function mutations, whereas gain-of-function mutations that could reveal genes with functional redundancy will be relatively rare. Another method of genetic screening in *Drosophila* has been developed that focuses specifically on systematic gain-of-function genetic screens (Rorth *et al.*, Development (1998) 125:1049-1057). This method is based on a modular mis-expression system utilizing components of the GAL4/UAS system (described above) where a modified P element, termed an "enhanced P" (EP) element, is genetically engineered to contain a GAL4-responsive UAS element and promoter. Any other transposons can also be used for this system. The resulting transposon is used to randomly tag genes by insertional mutagenesis (similar to the method of P element mutagenesis described above). Thousands of transgenic *Drosophila* strains, termed EP lines, can be generated, each containing a specific UAS-tagged gene. This approach takes advantage of the preference of P elements to insert at the 5'-ends of genes. Consequently, many of the genes that are tagged by insertion of EP elements become operably fused to a GAL4-regulated promoter, and increased expression or mis-expression of the randomly tagged gene can be induced by crossing in a GAL4 driver gene.

Systematic gain-of-function genetic screens for modifiers of phenotypes induced by mutation or mis-expression of a p53 gene can be performed by crossing several thousand *Drosophila* EP lines individually into a genetic background containing a mutant or mis-expressed p53 gene, and further containing an appropriate GAL4 driver transgene. It is also possible to remobilize the EP elements to obtain novel insertions. The progeny of these crosses are then analyzed for enhancement or suppression of the original mutant phenotype as described above. Those identified as having mutations that interact with the p53 gene can be tested further to verify the reproducibility and specificity of this genetic interaction. EP insertions that demonstrate a specific genetic interaction with a mutant or mis-expressed

p53 gene, have a physically tagged new gene which can be identified and sequenced using PCR or hybridization screening methods, allowing the isolation of the genomic DNA adjacent to the position of the EP element insertion.

5 **Identification of Molecules that Interact With p53**

A variety of methods can be used to identify or screen for molecules, such as proteins or other molecules, that interact with p53 protein, or derivatives or fragments thereof. The assays may employ purified p53 protein, or cell lines or a model organism such as *Drosophila* that has been genetically engineered to express p53 protein. Suitable screening methodologies are well known in the art to test for proteins and other molecules that interact with a gene/protein of interest (see *e.g.*, PCT International Publication No. WO 96/34099). The newly identified interacting molecules may provide new targets for pharmaceutical agents. Any of a variety of exogenous molecules, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides, or phage display libraries), may be screened for binding capacity. In a typical binding experiment, the p53 protein or fragment is mixed with candidate molecules under conditions conducive to binding, sufficient time is allowed for any binding to occur, and assays are performed to test for bound complexes. A variety of assays to find interacting proteins are known in the art, for example, immunoprecipitation with an antibody that binds to the protein in a complex followed by analysis by size fractionation of the immunoprecipitated proteins (*e.g.* by denaturing or nondenaturing polyacrylamide gel electrophoresis), Western analysis, non-denaturing gel electrophoresis, *etc.*

Two-hybrid assay systems

A preferred method for identifying interacting proteins is a two-hybrid assay system or variation thereof (Fields and Song, *Nature* (1989) 340:245-246; U.S. Pat. No. 5,283,173; for review see Brent and Finley, *Annu. Rev. Genet.* (1997) 31:663-704). The most commonly used two-hybrid screen system is performed using yeast. All systems share three elements: 1) a gene that directs the synthesis of a "bait" protein fused to a DNA binding domain; 2) one or more "reporter" genes having an upstream binding site for the bait, and 3) a gene that directs the synthesis of a "prey" protein fused to an activation domain that activates transcription of the reporter gene. For the screening of proteins that interact with p53 protein, the "bait" is preferably a p53 protein, expressed as a fusion protein to a DNA binding domain; and the "prey" protein is a protein to be tested for ability to interact with the bait, and is expressed as a fusion protein to a transcription activation

domain. The prey proteins can be obtained from recombinant biological libraries expressing random peptides.

The bait fusion protein can be constructed using any suitable DNA binding domain, such as the *E. coli* LexA repressor protein, or the yeast GAL4 protein (Bartel *et al.*, BioTechniques (1993) 14:920-924, Chasman *et al.*, Mol. Cell. Biol. (1989) 9:4746-4749; 5 Ma *et al.*, Cell (1987) 48:847-853; Ptashne *et al.*, Nature (1990) 346:329-331). The prey fusion protein can be constructed using any suitable activation domain such as GAL4, VP-16, *etc.* The preys may contain useful moieties such as nuclear localization signals (Ylikomi *et al.*, EMBO J. (1992) 11:3681-3694; Dingwall and Laskey, Trends Biochem. 10 Sci. Trends Biochem. Sci. (1991) 16:479-481) or epitope tags (Allen *et al.*, Trends Biochem. Sci. Trends Biochem. Sci. (1995) 20:511-516) to facilitate isolation of the encoded proteins. Any reporter gene can be used that has a detectable phenotype such as reporter genes that allow cells expressing them to be selected by growth on appropriate medium (*e.g.* HIS3, LEU2 described by Chien *et al.*, PNAS (1991) 88:9572-9582; and 15 Gyuris *et al.*, Cell (1993) 75:791-803). Other reporter genes, such as LacZ and GFP, allow cells expressing them to be visually screened (Chien *et al.*, *supra*).

Although the preferred host for two-hybrid screening is the yeast, the host cell in which the interaction assay and transcription of the reporter gene occurs can be any cell, such as mammalian (*e.g.* monkey, mouse, rat, human, bovine), chicken, bacterial, or insect 20 cells. Various vectors and host strains for expression of the two fusion protein populations in yeast can be used (U.S. Pat. No. 5,468,614; Bartel *et al.*, Cellular Interactions in Development (1993) Hartley, ed., Practical Approach Series xviii, IRL Press at Oxford University Press, New York, NY, pp. 153-179; and Fields and Sternglanz, Trends In Genetics (1994) 10:286-292). As an example of a mammalian system, interaction of 25 activation tagged VP16 derivatives with a GAL4-derived bait drives expression of reporters that direct the synthesis of hygromycin B phosphotransferase, chloramphenicol acetyltransferase, or CD4 cell surface antigen (Fearon *et al.*, PNAS (1992) 89:7958-7962). As another example, interaction of VP16-tagged derivatives with GAL4-derived baits drives the synthesis of SV40 T antigen, which in turn promotes the replication of the prey 30 plasmid, which carries an SV40 origin (Vasavada *et al.*, PNAS (1991) 88:10686-10690).

Typically, the bait p53 gene and the prey library of chimeric genes are combined by mating the two yeast strains on solid or liquid media for a period of approximately 6-8 hours. The resulting diploids contain both kinds of chimeric genes, i.e., the DNA-binding domain fusion and the activation domain fusion. Transcription of the reporter gene can be

detected by a linked replication assay in the case of SV40 T antigen (Vasavada *et al.*, *supra*) or using immunoassay methods (Alam and Cook, Anal. Biochem. (1990)188:245-254).

The activation of other reporter genes like URA3, HIS3, LYS2, or LEU2 enables the cells to grow in the absence of uracil, histidine, lysine, or leucine, respectively, and hence serves
 5 as a selectable marker. Other types of reporters are monitored by measuring a detectable signal. For example, GFP and lacZ have gene products that are fluorescent and chromogenic, respectively.

After interacting proteins have been identified, the DNA sequences encoding the proteins can be isolated. In one method, the activation domain sequences or DNA-binding
 10 domain sequences (depending on the prey hybrid used) are amplified, for example, by PCR using pairs of oligonucleotide primers specific for the coding region of the DNA binding domain or activation domain. If a shuttle (yeast to *E. coli*) vector is used to express the fusion proteins, the DNA sequences encoding the proteins can be isolated by transformation of *E. coli* using the yeast DNA and recovering the plasmids from *E. coli*. Alternatively, the
 15 yeast vector can be isolated, and the insert encoding the fusion protein subcloned into a bacterial expression vector, for growth of the plasmid in *E. coli*.

Antibodies and Immunoassay

p53 proteins encoded by any of SEQ ID NOs:2, 4, 6, 8, or 10 and derivatives and fragments thereof, such as those discussed above, may be used as an immunogen to
 20 generate monoclonal or polyclonal antibodies and antibody fragments or derivatives (*e.g.* chimeric, single chain, Fab fragments). For example, fragments of a p53 protein, preferably those identified as hydrophilic, are used as immunogens for antibody production using art-known methods such as by hybridomas; production of monoclonal antibodies in germ-free animals (PCT/US90/02545); the use of human hybridomas (Cole *et al.*, PNAS (1983)
 25 80:2026-2030; Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy (1985) Alan R. Liss, pp. 77-96), and production of humanized antibodies (Jones *et al.*, Nature (1986) 321:522-525; U.S. Pat. 5,530,101). In a particular embodiment, p53 polypeptide fragments provide specific antigens and/or immunogens, especially when coupled to carrier proteins. For example, peptides are covalently coupled to keyhole limpet antigen (KLH) and the
 30 conjugate is emulsified in Freund's complete adjuvant. Laboratory rabbits are immunized according to conventional protocol and bled. The presence of specific antibodies is assayed by solid phase immunosorbent assays using immobilized corresponding polypeptide. Specific activity or function of the antibodies produced may be determined by convenient *in vitro*, cell-based, or *in vivo* assays: *e.g.* *in vitro* binding assays, *etc.* Binding affinity may be

assayed by determination of equilibrium constants of antigen-antibody association (usually at least about 10^7 M^{-1} , preferably at least about 10^8 M^{-1} , more preferably at least about 10^9 M^{-1}). Example 11 below further describes the generation of anti-DMp53 antibodies.

Immunoassays can be used to identify proteins that interact with or bind to p53 protein. Various assays are available for testing the ability of a protein to bind to or compete with binding to a wild-type p53 protein or for binding to an anti-p53 protein antibody. Suitable assays include radioimmunoassays, ELISA (enzyme linked immunosorbent assay), immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (e.g., using colloidal gold, enzyme or radioisotope labels), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, etc.

Identification of Potential Drug Targets

Once new p53 genes or p53 interacting genes are identified, they can be assessed as potential drug or pesticide targets using animal models such as *Drosophila* or other insects, or using cells that express endogenous p53, or that have been engineered to express p53.

Assays of Compounds on Insects

Potential insecticidal compounds can be administered to insects in a variety of ways, including orally (including addition to synthetic diet, application to plants or prey to be consumed by the test organism), topically (including spraying, direct application of compound to animal, allowing animal to contact a treated surface), or by injection. Insecticides are typically very hydrophobic molecules and must commonly be dissolved in organic solvents, which are allowed to evaporate in the case of methanol or acetone, or at low concentrations can be included to facilitate uptake (ethanol, dimethyl sulfoxide).

The first step in an insect assay is usually the determination of the minimal lethal dose (MLD) on the insects after a chronic exposure to the compounds. The compounds are usually diluted in DMSO, and applied to the food surface bearing 0-48 hour old embryos and larvae. In addition to MLD, this step allows the determination of the fraction of eggs that hatch, behavior of the larvae, such as how they move /feed compared to untreated larvae, the fraction that survive to pupate, and the fraction that eclose (emergence of the adult insect from puparium). Based on these results more detailed assays with shorter exposure times may be designed, and larvae might be dissected to look for obvious

morphological defects. Once the MLD is determined, more specific acute and chronic assays can be designed.

In a typical acute assay, compounds are applied to the food surface for embryos, larvae, or adults, and the animals are observed after 2 hours and after an overnight incubation. For application on embryos, defects in development and the percent that survive to adulthood are determined. For larvae, defects in behavior, locomotion, and molting may be observed. For application on adults, behavior and neurological defects are observed, and effects on fertility are noted. Any deleterious effect on insect survival, motility and fertility indicates that the compound has utility in controlling pests.

For a chronic exposure assay, adults are placed on vials containing the compounds for 48 hours, then transferred to a clean container and observed for fertility, neurological defects, and death.

Assay of Compounds using Cell Cultures

Compounds that modulate (*e.g.* block or enhance) p53 activity may be tested on cells expressing endogenous normal or mutant p53s, and/or on cells transfected with vectors that express p53, or derivatives or fragments of p53. The compounds are added at varying concentration and their ability to modulate the activity of p53 genes is determined using any of the assays for tumor suppressor genes described above (*e.g.* by measuring transcription activity, apoptosis, proliferation/cell cycle, and/or transformation). Compounds that selectively modulate p53 are identified as potential drug candidates having p53 specificity.

Identification of small molecules and compounds as potential pharmaceutical compounds from large chemical libraries requires high-throughput screening (HTS) methods (Bolger, *Drug Discovery Today* (1999) 4:251-253). Several of the assays mentioned herein can lend themselves to such screening methods. For example, cells or cell lines expressing wild type or mutant p53 protein or its fragments, and a reporter gene can be subjected to compounds of interest, and depending on the reporter genes, interactions can be measured using a variety of methods such as color detection, fluorescence detection (*e.g.* GFP), autoradiography, scintillation analysis, *etc.*

Agricultural uses of insect p53 sequences

Insect p53 genes may be used in controlling agriculturally important pest species. For example, the proteins, genes, and RNAs disclosed herein, or their fragments may have activity in modifying the growth, feeding and/or reproduction of crop-damaging insects, or insect pests of farm animals or of other animals. In general, effective pesticides exert a

disabling activity on the target pest such as lethality, sterility, paralysis, blocked development, or cessation of feeding. Such pests include egg, larval, juvenile and adult forms of flies, mosquitos, fleas, moths, beetles, cicadia, grasshoppers, aphids and crickets. The functional analyses of insect p53 genes described herein has revealed roles for these genes and proteins in controlling apoptosis, response to DNA damaging agents, and protection of cells of the germline. Since overexpression of DMp53 induces apoptosis in *Drosophila*, the insect p53 genes and proteins in an activated form have application as "cell death" genes which if delivered to or expressed in specific target tissues such as the gut, nervous system, or gonad, would have a use in controlling insect pests. Alternatively, since DMp53 plays a role in response to DNA damaging agents such as X-rays, interference with p53 function in insects has application in sensitizing insects to DNA damaging agents for sterilization. For example, current methods for controlling pest populations through the release of irradiated insects into the environment (Knippling, J Econ Ent (1955) 48: 459-462; Knippling (1979) U.S. Dept. Agric. Handbook No. 512) could be improved by causing expression of dominant negative forms of p53 genes, proteins, or RNAs in insects and most preferably germline tissue of insects, or by exposing insects to chemical compounds which block p53 function.

Mutational analysis of insect p53 proteins may also be used in connection with the control of agriculturally-important pests. In this regard, mutational analysis of insect p53 genes provides a rational approach to determine the precise biological function of this class of proteins in invertebrates. Further, mutational analysis coupled with large-scale systematic genetic modifier screens provides a means to identify and validate other potential pesticide targets that might be constituents of the p53 signaling pathway. Tests for pesticidal activities can be any method known in the art. Pesticides comprising the nucleic acids of the insect p53 proteins may be prepared in a suitable vector for delivery to a plant or animal. Such vectors include *Agrobacterium tumefaciens* Ti plasmid-based vectors for the generation of transgenic plants (Horsch *et al.*, Proc Natl Acad Sci U S A. (1986) 83(8):2571-2575; Fraley *et al.*, Proc. Natl. Acad. Sci. USA (1983) 80:4803) or recombinant cauliflower mosaic virus for the inoculation of plant cells or plants (U.S. Pat No. 4,407,956); retrovirus based vectors for the introduction of genes into vertebrate animals (Burns *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90:8033-37); and vectors based on transposable elements for incorporation into invertebrate animals using vectors and methods already described above. For example, transgenic insects can be generated using a transgene comprising a p53 gene operably fused to an appropriate inducible promoter, such

as a tTA-responsive promoter, in order to direct expression of the tumor suppressor protein at an appropriate time in the life cycle of the insect. In this way, one may test efficacy as an insecticide in, for example, the larval phase of the life cycle (*e.g.*, when feeding does the greatest damage to crops).

5 Recombinant or synthetic p53 proteins, RNAs or their fragments, in wild-type or mutant forms, can be assayed for insecticidal activity by injection of solutions of p53 proteins or RNAs into the hemolymph of insect larvae (Blackburn, *et al.*, Appl. Environ. Microbiol. (1998) 64(8):3036-41; Bowen and Ensign, Appl. Environ. Microbiol. (1998) 64(8):3029-35). Further, transgenic plants that express p53 proteins or RNAs or their
10 fragments can be tested for activity against insect pests (Estruch *et al.*, Nat. Biotechnol. (1997) 15(2):137-41).

Insect p53 genes may be used as insect control agents in the form of recombinant viruses that direct the expression of a tumor suppressor gene in the target pest. A variety of suitable recombinant virus systems for expression of proteins in infected insect cells are
15 well known in the art. A preferred system uses recombinant baculoviruses. The use of recombinant baculoviruses as a means to engineer expression of toxic proteins in insects, and as insect control agents, has a number of specific advantages including host specificity, environmental safety, the availability of vector systems, and the potential use of the recombinant virus directly as a pesticide without the need for purification or formulation of
20 the tumor suppressor protein (Cory and Bishop, Mol. Biotechnol. (1997) 7(3):303-13; and U.S. Pat. Nos. 5,470,735; 5,352,451; 5,770,192; 5,759,809; 5,665,349; and 5,554,592). Thus, recombinant baculoviruses that direct the expression of insect p53 genes can be used for both testing the pesticidal activity of tumor suppressor proteins under controlled laboratory conditions, and as insect control agents in the field. One disadvantage of wild
25 type baculoviruses as insect control agents can be the amount of time between application of the virus and death of the target insect, typically one to two weeks. During this period, the insect larvae continue to feed and damage crops. Consequently, there is a need to develop improved baculovirus-derived insect control agents which result in a rapid cessation of feeding of infected target insects. The cell cycle and apoptotic regulatory roles
30 of p53 in vertebrates raises the possibility that expression of tumor suppressor proteins from recombinant baculovirus in infected insects may have a desirable effect in controlling metabolism and limiting feeding of insect pests.

Insect p53 genes, RNAs, proteins or fragments may be formulated with any carrier suitable for agricultural use, such as water, organic solvents and/or inorganic solvents. The

pesticide composition may be in the form of a solid or liquid composition and may be prepared by fundamental formulation processes such as dissolving, mixing, milling, granulating, and dispersing. Compositions may contain an insect p53 protein or gene in a mixture with agriculturally acceptable excipients such as vehicles, carriers, binders, UV blockers, adhesives, hemecants, thickeners, dispersing agents, preservatives and insect attractants. Thus the compositions of the invention may, for example, be formulated as a solid comprising the active agent and a finely divided solid carrier. Alternatively, the active agent may be contained in liquid compositions including dispersions, emulsions and suspensions thereof. Any suitable final formulation may be used, including for example, granules, powder, bait pellets (a solid composition containing the active agent and an insect attractant or food substance), microcapsules, water dispersible granules, emulsions and emulsified concentrates. Examples of adjuvant or carriers suitable for use with the present invention include water, organic solvent, inorganic solvent, talc, pyrophyllite, synthetic fine silica, attapugus clay, kieselguhr chalk, diatomaceous earth, lime, calcium carbonate, bontonite, fuller's earth, cottonseed hulls, wheat flour, soybean flour, pumice, tripoli, wood flour, walnut shell flour, redwood flour, and lignin. The compositions may also include conventional insecticidal agents and/or may be applied in conjunction with conventional insecticidal agents.

20 **EXAMPLES**

The following examples describe the isolation and cloning of the nucleic acid sequence of SEQ ID NOs: 1, 3, 5, 7, 9, and 18, and how these sequences, derivatives and fragments thereof, and gene products can be used for genetic studies to elucidate mechanisms of the p53 pathway as well as the discovery of potential pharmaceutical agents that interact with the pathway.

These Examples are provided merely as illustrative of various aspects of the invention and should not be construed to limit the invention in any way.

Example 1: Preparation of *Drosophila* cDNA Library

30 A *Drosophila* expressed sequence tag (EST) cDNA library was prepared as follows. Tissue from mixed stage embryos (0-20 hour), imaginal disks and adult fly heads were collected and total RNA was prepared. Mitochondrial rRNA was removed from the total RNA by hybridization with biotinylated rRNA specific oligonucleotides and the resulting RNA was selected for polyadenylated mRNA. The resulting material was then used to

construct a random primed library. First strand cDNA synthesis was primed using a six nucleotide random primer. The first strand cDNA was then tailed with terminal transferase to add approximately 15 dGTP molecules. The second strand was primed using a primer which contained a NotI site followed by a 13 nucleotide C-tail to hybridize to the G-tailed first strand cDNA. The double stranded cDNA was ligated with BstXI adaptors and digested with NotI. The cDNA was then fractionated by size by electrophoresis on an agarose gel and the cDNA greater than 700 bp was purified. The cDNA was ligated with NotI, BstXI digested pCDNA-sk+ vector (a derivative of pBluescript, Stratagene) and used to transform *E. coli* (XL1blue). The final complexity of the library was 6×10^6 independent clones.

The cDNA library was normalized using a modification of the method described by Bonaldo *et al.* (Genome Research (1996) 6:791-806). Biotinylated driver was prepared from the cDNA by PCR amplification of the inserts and allowed to hybridize with single stranded plasmids of the same library. The resulting double-stranded forms were removed using streptavidin magnetic beads, the remaining single stranded plasmids were converted to double stranded molecules using Sequenase (Amersham, Arlington Hills, IL), and the plasmid DNA stored at -20°C prior to transformation. Aliquots of the normalized plasmid library were used to transform *E. coli* (XL1blue or DH10B), plated at moderate density, and the colonies picked into a 384-well master plate containing bacterial growth media using a Qbot robot (Genetix, Christchurch, UK). The clones were allowed to grow for 24 hours at 37° C then the master plates were frozen at -80° C for storage. The total number of colonies picked for sequencing from the normalized library was 240,000. The master plates were used to inoculate media for growth and preparation of DNA for use as template in sequencing reactions. The reactions were primarily carried out with primer that initiated at the 5' end of the cDNA inserts. However, a minor percentage of the clones were also sequenced from the 3' end. Clones were selected for 3' end sequencing based on either further biological interest or the selection of clones that could extend assemblies of contiguous sequences ("contigs") as discussed below. DNA sequencing was carried out using ABI377 automated sequencers and used either ABI FS, dirhodamine or BigDye chemistries (Applied Biosystems, Inc., Foster City, CA).

Analysis of sequences was done as follows: the traces generated by the automated sequencers were base-called using the program "Phred" (Gordon, Genome Res. (1998) 8:195-202), which also assigned quality values to each base. The resulting sequences were

trimmed for quality in view of the assigned scores. Vector sequences were also removed. Each sequence was compared to all other fly EST sequences using the BLAST program and a filter to identify regions of near 100% identity. Sequences with potential overlap were then assembled into contigs using the programs "Phrap", "Phred" and "Consed" (Phil
5 Green, University of Washington, Seattle, Washington;
<http://bozeman.mbt.washington.edu/phrap.docs/phrap.html>). The resulting assemblies were then compared to existing public databases and homology to known proteins was then used to direct translation of the consensus sequence. Where no BLAST homology was available, the statistically most likely translation based on codon and hexanucleotide preference was
10 used. The Pfam (Bateman *et al.*, Nucleic Acids Res. (1999) 27:260-262) and Prosite (Hofmann *et al.*, Nucleic Acids Res. (1999) 27(1):215-219) collections of protein domains were used to identify motifs in the resulting translations. The contig sequences were archived in an Oracle-based relational database (FlyTag™, Exelixis Pharmaceuticals, Inc., South San Francisco, CA).

15

Example 2: Other cDNA libraries

A *Leptinotarsa* (Colorado Potato Beetle) library was prepared using the Lambda ZAP cDNA cloning kit from Stratagene (Stratagene, La Jolla, CA, cat#200450), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt
20 primed using mRNA from mixed stage larvae *Leptinotarsa*.

A *Tribolium* library was made using pSPORT cDNA library construction system (Life Technologies, Gaithersburg, MD), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt primed using mRNA from adult *Tribolium*.

Example 3: Cloning of the p53 nucleic acid from *Drosophila* (DMp53)

The TBLASTN program (Altschul *et al.*, *supra*) was used to query the FlyTag™ database with a squid p53 protein sequence (GenBank gi:1244762), chosen because the squid sequence was one of only two members of the p53 family that had been identified previously from an invertebrate. The results revealed a single sequence contig, which was
30 960 bp in length and which exhibited highly significant homology to squid p53 (score=192, $P=5.1 \times 10^{-12}$). Further analysis of this sequence with the BLASTX program against GenBank protein sequences demonstrated that this contig exhibited significant homology to the entire known family of p53-like sequences in vertebrates, and that it contained coding

sequences homologous to the p53 family that encompassed essentially all of the DNA-binding domain, which is the most conserved region of the p53 protein family. Inspection of this contig indicated that it was an incomplete cDNA, missing coding regions C-terminal to the presumptive DNA-binding domain as well as the 3' untranslated region of the mRNA.

5 The full-length cDNA clone was produced by Rapid Amplification of cDNA ends (RACE: Frohman *et al.*, PNAS (1988) 85:8998-9002). A RACE-ready library was generated from Clontech (Palo Alto, CA) *Drosophila* embryo poly A⁺ RNA (Cat#694-1) using Clontech's Marathon cDNA amplification kit (Cat# K1802), and following manufacturer's directions. The following primers were used on the library to retrieve full-
10 length clones:

3'373	CCATGCTGAAGCAATAACCACCGATG	SEQ ID NO:11
3'510	GGAACACACGCAAATTAAGTGGTTGGATGG	SEQ ID NO:12
3'566	TGATTTTGACAGCGGACCGGG	SEQ ID NO:13
15 3'799	GGAAGTTTCTTTTCGCCCCGATACACGAG	SEQ ID NO:14
5'164	GGCACAAAGAAAGCACTGATTCCGAGG	SEQ ID NO:15
5'300	GGAATCTGATGCAGTTCAGCCAGCAATC	SEQ ID NO:16
5'932	GGATCGCATCCAAGACGAACGCC	SEQ ID NO:17

20 RACE reactions to obtain additional 5' and 3' sequence of the *Drosophila* p53 cDNA were performed as follows. Each RACE reaction contained: 40 µl of H₂O, 5 µl of 10X Advantage PCR buffer (Clontech), 1 µl of specific p53 RACE primer at 10 µM, 1 µl of API primer (from Clontech Marathon kit) at 10 µM, 1 µl of cDNA, 1 µl of dNTPs at 5 mM, 1 µl of Advantage DNA polymerase (Clontech). For 5' RACE, the reactions
25 contained either the 3'373, 3'510, 3'566, or 3'799 primers. For 3' RACE, the reactions contained either the 5'164 or 5'300 primers. The reaction mixtures were subjected to the following thermocycling program steps for touchdown PCR: (1) 94°C 1 min, (2) 94°C 0.5 min, (3) 72°C 4 min, (4) repeat steps 2-3 four times, (5) 94°C 0.5 min, (6) 70°C 4 min, (7) repeat steps 5-6 four times, (8) 94°C 0.33 min, (9) 68°C 4 min, (10) repeat steps 8-9 24
30 times, (11) 68°C 4 min, (12) remain at 4°C.

Products of the RACE reactions were analyzed by gel electrophoresis. Discrete DNA species of the following sizes were observed in the RACE products produced with each of the following primers: 3'373, approx. 400 bp; 3'510, approx. 550 bp, 3'566, approx. 600 bp; 3'799, approx. 850 bp; 5'164, approx. 1400 bp, 5'300 approx. 1300 bp. The RACE

DNA products were cloned directly into the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, California) following the manufacturers directions. Colonies of transformed *E. coli* were picked for each construct, and plasmid DNA prepared using a QIAGEN tip 20 kit (QIAGEN, Valencia, California). Sequences of the RACE cDNA
5 inserts in within each clone were determined using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc. Foster City, California) and either M13 reverse or BigT7 primers for priming from flanking vector sequences, or 5'932 or 3'373 primers (described above) for priming internally from *Drosophila* p53 cDNA sequences. The products were analyzed using ABI 377 DNA sequencer. Sequences were assembled
10 into a contig using the Sequencher program (Gene Codes Corporation), and contained a single open reading frame encoding a predicted protein of 385 amino acids, which compared favorably with the known lengths of vertebrate p53 proteins, 363 to 396 amino acids (Soussi *et al.*, Oncogene (1990) 5:945-952). Analysis of the predicted *Drosophila* p53 protein using the BLASTP homology searching program and the GenBank database
15 confirmed that this protein was a member of the p53 family, since it exhibited highly significant homology to all known p53 related proteins, but no significant homology to other protein families.

Example 4: Cloning of p53 Nucleic Acid Sequences from other insects

20 The PCR conditions used for cloning the p53 nucleic acid sequences comprised a denaturation step of 94° C, 5 min; followed by 35 cycles of: 94° C 1 min, 55° C 1 min 72° C 1 min; then, a final extension at 72° C 10 min. All DNA sequencing reactions were performed using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc.) and products were analyzed using ABI 377 DNA sequencers. Trace data
25 obtained from the ABI 377 DNA sequencers was analyzed and assembled into contigs using the Phred-Phrap programs.

The DMp53 DNA and protein sequences were used to query sequences from *Tribolium*, *Leptinotarsa*, and *Heliothis* cDNA libraries using the BLAST computer program, and the results revealed several candidate cDNA clones that might encode p53
30 related sequences. For each candidate p53 cDNA clone, well-separated, single colonies were streaked on a plate and end-sequenced to verify the clones. Single colonies were picked and the plasmid DNA was purified using Qiagen REAL Preps (Qiagen, Inc., Valencia, CA). Samples were then digested with appropriate enzymes to excise insert from

vector and determine size. For example, the vector pOT2, (www.fruitfly.org/EST/pOT2vector.html) can be excised with XhoI/EcoRI; or pBluescript (Stratagene) can be excised with BssH II. Clones were then sequenced using a combination of primer walking and *in vitro* transposon tagging strategies.

5 For primer walking, primers were designed to the known DNA sequences in the clones, using the Primer-3 software (Steve Rozen, Helen J. Skaletsky (1998) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). These primers were then used in sequencing reactions to extend the sequence until the full sequence of the insert was determined.

10 The GPS-1 Genome Priming System *in vitro* transposon kit (New England Biolabs, Inc., Beverly, MA) was used for transposon-based sequencing, following manufacturer's protocols. Briefly, multiple DNA templates with randomly interspersed primer-binding sites were generated. These clones were prepared by picking 24 colonies/clone into a Qiagen REAL Prep to purify DNA and sequenced by using supplied primers to perform
15 bidirectional sequencing from both ends of transposon insertion.

Sequences were then assembled using Phred/Phrap and analyzed using Consed. Ambiguities in the sequence were resolved by resequencing several clones. This effort resulted in several contiguous nucleotide sequences. For *Leptinotarsa*, a contig was assembled of 2601 bases in length, encompassing an open reading frame (ORF) of 1059
20 nucleotides encoding a predicted protein of 353 amino acids. The ORF extends from base 121-1180 of SEQ ID NO:3. For *Tribolium*, a contig was assembled of 1292 bases in length, encompassing an ORF of 1050 nucleotides, extending from base 95-1145 of SEQ ID NO:5, and encoding a predicted protein of 350 amino acids. The analysis of another candidate *Tribolium* p53 clone also generated a second contig of 509 bases in length, encompassing a
25 partial ORF of 509 nucleotides (SEQ ID NO: 7), and encoding a partial protein of 170 amino acids. For *Heliothis*, a contig was assembled of 434 bases in length, encompassing a partial ORF of 434 nucleotides (SEQ ID NO:9), and encoding a partial protein of 145 amino acids.

30 **Example 5: Northern Blot analysis of DMp53**

Northern blot analysis using standard methods was performed using three different poly(A)+ mRNA preparations. 0-12 h embryo, 12-24 h embryo, and adult, which were fractionated on an agarose gel along with size standards and blotted to a nylon membrane. A DNA fragment containing the entire *Drosophila* p53 coding region was excised by

HincII digestion, separated by electrophoresis in an agarose gel, extracted from the gel, and ³²P-labeled by random-priming using the Rediprime labeling system (Amersham, Piscataway, NJ). Hybridization of the labeled probe to the mRNA blot was performed overnight. The blot was washed at high stringency (0.2x SSC/0.1% SDS at 65°C) and mRNA species that specifically hybridized to the probe were detected by autoradiography using X-ray film. The results showed a single cross-hybridizing mRNA species of approximately 1.6 kilobases in all three mRNA sources. This data was consistent with the observed sizes of the 5' and 3' RACE products described above.

10 **Example 6: Cytogenetic mapping of the DMp53 gene**

It was of interest to identify the map location of the DMp53 gene in order to determine whether any existing *Drosophila* mutants correspond to mutations in the DMp53 gene, as well as for engineering new mutations within this gene. The cytogenetic location of the DMp53 gene was determined by *in situ* hybridization to polytene chromosomes (Pardue, Meth Cell Biol (1994) 44:333-351) following the protocol outlined below (steps A-C).

(A) Preparation of polytene chromosome squashes: Dissected salivary glands were placed into a drop of 45% acetic acid. Glands were transferred to drop of 1:2:3 mixture of lactic acid: water:acetic acid. Glands were then squashed between a cover slip and a slide and incubated at 4°C overnight. Squashes were frozen in liquid N₂ and the coverslip removed. Slides were then immediately immersed in 70% ethanol for 10 min. and then air dried. Slides were then heat treated for 30 min. at 68°C in 2x SSC buffer. Squashes were then dehydrated by treatment with 70% ethanol for 10 min. followed by 95% ethanol for 5 min.

(B) Preparation of a biotinylated hybridization probe: a solution was prepared by mixing: 50 µl of 1 M Tris-HCl pH 7.5, 6.35 µl of 1 M MgCl₂, 0.85 µl of beta-mercaptoethanol, 0.625 µl of 100 mM dATP, 0.625 µl of 100 mM dCTP, 0.625 µl of 100 mM dGTP, 125 µl of 2 M HEPES pH 6.6, and 75 µl of 10 mg/ml pd(N)₆ (Pharmacia, Kalamazoo, MI). 10 µl of this solution was then mixed with 2 µl 10 mg/ml bovine serum albumin, 33 µl containing (0.5 µg) DMp53 cDNA fragment denatured by quick boiling, 5 µl of 1 mM biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN), and 1 µl of Klenow DNA polymerase (2 U) (Boehringer Mannheim). The mixture was incubated at room temperature overnight and the following components were then added: 1 µl of 1 mg/ml sonicated denatured salmon sperm DNA, 5.5 µl 3 M sodium acetate pH 5.2, and 150 µl

ethanol (100%). After mixing the solution was stored at -70°C for 1-2 hr. DNA precipitate was collected by centrifugation in a microcentrifuge and the pellet was washed once in 70% ethanol, dried in a vacuum, dissolved in 50 µl TE buffer, and stored at -20°C.

(C) Hybridization and staining was performed as follows: 20 µl of the probe added to a hybridization solution (112.5 µl formamide; 25 µl 20x SSC, pH 7.0; 50 µl 50% dextran sulfate; 62.5 µl distilled H₂O) was placed on the squash. A coverslip (22 mm²) was placed on the squash and sealed with rubber cement and placed on the airtight moist chamber overnight at 42°C. Rubber cement was removed by peeling off cement, then coverslip removed in 2x SSC buffer at 37°C. Slides were washed twice 15 min each in 2x SSC buffer at 37°C. Slides were then washed twice 15 min each in PBS buffer at room temperature. A mixture of the following "Elite" solution was prepared by mixing: 1 ml of PBT buffer (PBS buffer with 0.1% Tween 20), 10 µl of Vectastain A (Vector Laboratories, Burlingame, CA), and 10 µl of Vectastain B (Vector Laboratories). The mixture was then allowed to incubate for 30 min. 50 µl of the Elite solution was added to the slide then drained off. 75 µl of the Elite solution was added to slide and a coverslip was placed onto the slide. The slide was incubated in moist chamber 1.5-2 hr at 37°C. The coverslip was then removed in PBS buffer, and the slide was washed twice 10 min each in PBS buffer.

A fresh solution of DAB (diaminobenzidine) in PBT buffer was made by mixing 1 µl of 0.3% hydrogen peroxide with 40 µl 0.5 mg/ml DAB solution. 40 µl of the DAB/peroxide solution was then placed onto each slide. A coverslip was placed onto the slide and incubated 2 min. Slides were then examined under a phase microscope and reaction was stopped in PBS buffer when signal was determined to be satisfactory. Slides were then rinsed in running H₂O for 10 min. and air dried. Finally, slides were inspected under a compound microscope to assign a chromosomal location to the hybridization signal. A single clear region of hybridization was observed on the polytene chromosome squashes which was assigned to cytogenetic bands 94D2-6.

Example 7: Isolation and sequence analysis of a genomic clone for the DMp53 gene

PCR was used to generate DNA probes for identification of genomic clones containing the DMp53 gene. Each reaction (50 µl total volume) contained 100 ng *Drosophila* genomic DNA, 2.5 µM each dNTP, 1.5 mM MgCl₂, 2 µM of each primer, and 1 µl of TAKARA exTaq DNA polymerase (PanVera Corp., Madison, WI). Reactions were set up with primer pair 5'164 & 3'510 (described above), and thermocycling conditions used were as follows (where 0:00 indicates time in minutes:seconds): initial denaturation of

94°C, 2:00; followed by 10 cycles of 94°C, 0:30, 58°C 0:30, 68°C, 4:00; followed by 20 cycles of 94°C, 0:30, 55°C, 0:30, 68°C, 4:00 + 0:20 per cycle. PCR products were then fractionated by agarose gel electrophoresis, ³²P-labeled by nick translation, and hybridized to nylon membranes containing high-density arrayed P1 clones from the Berkeley

5 *Drosophila* Genome Project (University of California, Berkeley, and purchased from Genome Systems, Inc., St. Louis, MO). Four positive P1 clones were identified: DS01201, DS02942, DS05102, and DS06254, and each clone was verified using a PCR assay with the primer pair described above. To prepare DNA for sequencing, *E. coli* containing each P1 clone was streaked to single colonies on LB agar plates containing 25 µg/ml kanamycin,

10 and grown overnight at 37°C. Well-separated colonies for each P1 clone were picked and used to inoculate 250 ml LB medium containing 25 µg/ml kanamycin and cultures were grown for 16 hours at 37°C with shaking. Bacterial cells were collected by centrifugation, and DNA purified with a Qiagen Maxi-Prep System kit (QIAGEN, Inc., Valencia, California). Genomic DNA sequence from the P1 clones was obtained using a strategy that

15 combined shotgun and directed sequencing of a small insert plasmid DNA library derived from the P1 clone DNAs (Ruddy *et al.* Genome Research (1997) 7:441-456). All DNA sequencing and analysis were performed as described before, and P1 sequence contigs were analyzed using the BLAST sequence homology searching programs to identify those that contained the DMp53 gene or other coding regions. This analysis demonstrated that the

20 DMp53 gene was divided into 8 exons and 7 introns. In addition, the BLAST analysis indicated the presence of two additional genes that flank the DMp53 gene; one exhibited homology to a human gene implicated in nephropathic cystinosis (labeled CTNS-like gene) and the second gene exhibited homology to a large family of oxidoreductases. Thus, we could operationally define the limits of the DMp53 gene as an 8,805 bp corresponding the

25 DNA region lying between the putative CTNS-like and oxidoreductase-like genes.

Example 8: Analysis of p53 Nucleic Acid Sequences

Upon completion of cloning, the sequences were analyzed using the Pfam and Prosite programs, and by visual analysis and comparison with other p53 sequences.

30 Regions of cDNA encoding the various domains of SEQ ID Nos 1-6 are depicted in Table I above. Additionally, Pfam predicted p53 similarity regions for the partial TRIB-Bp53 at amino acid residues 118-165 (SEQ ID NO:8) encoded by nucleotides 354-495 (SEQ ID NO:7), and for the partial HELIOp53 at amino acid residues 105-138 (SEQ ID NO:10) encoded by nucleotides 315-414 (SEQ ID NO:9).

Nucleotide and amino acid sequences for each of the p53 nucleic acid sequences and their encoded proteins were searched against all available nucleotide and amino acid sequences in the public databases, using BLAST (Altschul *et al.*, *supra*). Tables 2-6 below summarize the results. The 5 most similar sequences are listed for each p53 gene.

5

TABLE 2 – DMp53

DNA BLAST of SEQ ID NO:1	
GI#	DESCRIPTION
6664917= C019980	<i>Drosophila melanogaster</i> . *** SEQUENCING IN PROGRESS ***, in ordered pieces
5670489=AC008200	<i>Drosophila melanogaster</i> chromosome 3 clone BACR17P04 (D757) RPCI-98 17.P.4 map 94D-94E strain y; cn bw sp, *** SEQUENCING IN PROGRESS***, 70 unordered pieces.
4419483=AI516383	<i>Drosophila melanogaster</i> cDNA clone LD42237 5prime, mRNA sequence
4420516=AI517416	<i>Drosophila melanogaster</i> cDNA clone GH28349 5prime, mRNA sequence
4419333=AI516233	<i>Drosophila melanogaster</i> cDNA clone LD42031 5prime, mRNA sequence
PROTEIN BLAST of SEQ ID NO:2	
GI#	DESCRIPTION
1244764= AA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
1244762= AA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
2828704= AC31133	tumor protein p53 [<i>Xiphophorus helleri</i>]
2828706= AC31134	tumor protein p53 [<i>Xiphophorus maculatus</i>]
3695098= AC62643	DN p63 beta [<i>Mus musculus</i>]

TABLE 3 – CPBp53

DNA BLAST of SEQ ID NO:3	
GI#	DESCRIPTION
6468070= AC008132	<i>Homo sapiens</i> , complete sequence Chromosome 22q11 PAC Clone pac99506 In CES-DGCR Region
4493931= AL034556	<i>Plasmodium falciparum</i> MAL3P5, complete sequence
3738114= AC004617	<i>Homo sapiens</i> chromosome Y, clone 264,M.20, complete sequence
4150930= AC005083	<i>Homo sapiens</i> BAC clone CTA-281G5 from 7p15-p21, complete sequence
4006838= AC006079	<i>Homo sapiens</i> chromosome 17, clone hRPK.855_D_21, complete sequence
PROTEIN BLAST of SEQ ID NO:4	
GI#	DESCRIPTION
1244764= AA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
1244762= AA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
4530686=AA03817	unnamed protein product [<i>unidentified</i>]

4803651=CAA72225	P73 splice variant [<i>Cercopithecus aethiops</i>]
2370177=CAA72219	first splice variant [<i>Homo sapiens</i>]

TABLE 4 – TRIB-Ap53

DNA BLAST of SEQ ID NO:5	
GI#	DESCRIPTION
5877734=AW024204	wv01h01.x1 NCI_CGAP_Kid3 <i>Homo sapiens</i> cDNA clone IMAGE:2528305 3'. mRNA sequence
16555= X65053	<i>A.thaliana</i> mRNA for eukaryotic translation initiation factor 4A-2
6072079=AW101398	sd79d06.y1 Gm-c1009 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1009-612 5', mRNA sequence
6070492=AW099879	sd17g11.y2 Gm-c1012 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1012-2013 5', mRNA sequence
4105775= AF049919	<i>Petunia x hybrida</i> PGP35 (PGP35) mRNA, complete cds.
PROTEIN BLAST of SEQ ID NO:6	
GI#	DESCRIPTION
1244764=AAA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
3273745=AAC24830	p53 homolog [<i>Homo sapiens</i>]
1244762=AAA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
3695096=AAC62642	N p63 gamma [<i>Mus musculus</i>]
3695080=AAC62634	DN p63 gamma [<i>Homo sapiens</i>]

5

TABLE 5 – TRIB-Bp53

DNA BLAST of SEQ ID NO:7	
GI#	DESCRIPTION
4689085= AF043641	<i>Barbus barbus</i> p73 mRNA, complete cds
4530689= A64588	Sequence 7 from Patent WO9728186
N/A	No further homologies
PROTEIN BLAST of SEQ ID NO:8	
GI#	DESCRIPTION
4689086=AAD27752	p73 [<i>Barbus barbus</i>]
4530686=CAA03817	unnamed protein product [unidentified]
4803651=CAA72225	P73 splice variant [<i>Cercopithecus aethiops</i>]
4530690=CAA03819	unnamed protein product [unidentified]
4530684=CAA03816	unnamed protein product [unidentified]

TABLE 6 – HELIO p53

DNA BLAST of SEQ ID NO:9	
GI#	DESCRIPTION
N/A	No homologies found

PROTEIN BLAST of SEQ ID NO:10	
GI#	DESCRIPTION
2781308= 1YCSA	Chain A. p53-53bp2 Complex
1310770= 1TSRA	Chain A. p53 Core Domain In Complex With Dna
1310771= 1TSRB	Chain B. p53 Core Domain In Complex With Dna
1310772= 1TSRC	Chain C. p53 Core Domain In Complex With Dna
1310960= 1TUPA	Chain A. Tumor Suppressor p53 Complexed With Dna

BLAST analysis using each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous novel amino acids with respect to published sequences indicate the following: 7 amino acid residues for DMp53 and for
 5 TRIB-Ap53, 6 amino acid residues for CPBp53, and 5 amino acid residues for TRIB-Bp53 and HELIOp53.

BLAST results for each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous amino acids for which there are no sequences contained within public database sharing 100% sequence similarity indicate the
 10 following: 9 amino acid residues for DMp53, CPBp5, TRIB-Ap53, and TRIB-Bp53, and 6 amino acid residues for HELIOp53.

Example 9: *Drosophila* genetics

Fly culture and crosses were performed according to standard procedures at 22-25°C
 15 (Ashburner, *supra*). GI-DMp53 overexpression constructs were made by cloning a BclII HincII fragment spanning the DMp53 open reading frame into a vector (pExPress) containing glass multiple repeats upstream of a minimal heat shock promoter. The pExPress vector is an adapted version of the pGMR vector (Hay *et al.*, Development (1994) 120:2121-2129) which contains an alpha tubulin 3' UTR for increased protein stabilization
 20 and a modified multiple cloning site. Standard P-element mediated germ line transformation was used to generate transgenic lines containing these constructs (Rubin and Spradling, *supra*). For X-irradiation experiments, third instar larvae in vials were exposed to 4,000 Rads of X-rays using a Faxitron X-ray cabinet system (Wheeling, IL).

25 Example 10: Whole-mount RNA *in situ* hybridization, TUNEL, and Immunocytochemistry

In situ hybridization was performed using standard methods (Tautz and Pfeifle, Chromosoma (1989) 98:81-85). DMp53 anti-sense RNA probe was generated by digesting DMp53 cDNA with EcoRI and transcribing with T7 RNA polymerase. For

immunocytochemistry, third instar larval eye and wing discs were dissected in PBS, fixed in 2% formaldehyde for 30 minutes at room temperature, permeabilized in PBS+0.5% Triton for 15 minutes at room temperature, blocked in PBS+5% goat serum, and incubated with primary antibody for two hours at room temperature or overnight at 4°C. Anti-phospho-histone staining used Anti-phospho-histone H3 Mitosis Marker (Upstate Biotechnology, Lake Placid, NY) at a 1:500 dilution. Anti-DMp53 monoclonal antibody staining used hybridoma supernatant diluted 1:2. Goat anti-mouse or anti-rabbit secondary antibodies conjugated to FITC or Texas Red (Jackson ImmunoResearch, West Grove, PA) were used at a 1:200 dilution. Antibodies were diluted in PBS+5% goat serum. TUNEL assay was performed by using the Apoptag Direct kit (Oncor, Gaithersburg, MD) per manufacturer's protocol with a 0.5% Triton/PBS permeabilization step. Discs were mounted in anti-fade reagent (Molecular Probes, Eugene, OR) and images were obtained on a Leica confocal microscope. BrDU staining was performed as described (de Nooij *et al.*, Cell. (1996)87(7):1237-1247) and images were obtained on an Axioplan microscope (Zeiss, Thornwood, NY).

Example 11: Generation of anti-DMp53 antibodies

Anti-DMp53 rabbit polyclonal (Josman Labs, Napa, CA) and mouse monoclonal antibodies (Antibody Solutions Inc., Palo Alto, CA) were generated by standard methods using a full-length DMp53 protein fused to glutathione-S-transferase (GST-DMp53) as antigen. Inclusion bodies of GST-DMp53 were purified by centrifugation using B-PER buffer (Pierce, Rockford, IL) and injected subcutaneously into rabbits and mice for immunization. The final boost for mouse monoclonal antibody production used intravenous injection of soluble GST-DMp53 produced by solubilization of GST-DMp53 in 6M GuHCl and dialysis into phosphate buffer containing 1M NaCl. Hybridoma supernatants were screened by ELISA using a soluble 6XHIS-tagged DMp53 protein bound to Ni-NTA coated plates (Qiagen, Valencia, CA) and an anti-mouse IgG Fc-fragment specific secondary antibody.

Example 12: Functional analysis

The goal of this series of experiments was to compare and contrast the functions of the insect p53s to those of the human p53. The DMp53 was chosen to carry out this set of experiments, although any of the other insect p53s could be used as well.

p53 involvement in the cell death pathway

To determine whether DMp53 can serve the same functions in vivo as human p53, DMp53 was ectopically expressed in *Drosophila* larval eye discs using *glass*-responsive enhancer elements. The *glass*-DMp53 (gl-DMp53) transgene expresses DMp53 in all cells posterior to the morphogenetic furrow. During eye development, the morphogenetic furrow sweeps from the posterior to the anterior of the eye disc. Thus, gl-DMp53 larvae express DMp53 in a field of cells which expands from the posterior to the anterior of the eye disc during larval development.

Adult flies carrying the gl-DMp53 transgene were viable but had small, rough eyes with fused ommatidia (any of the numerous elements of the compound eye). TUNEL staining of gl-DMp53 eye discs showed that this phenotype was due, at least in part, to widespread apoptosis in cells expressing DMp53. Results were confirmed by the detection of apoptotic cells with acridine orange and Nile Blue. TUNEL-positive cells appeared within 15-25 cell diameters of the furrow. Given that the furrow moves approximately 10 cell diameters per hour, this indicated that the cells became apoptotic 2-3 hours after DMp53 was expressed. Surprisingly, co-expression of the baculovirus cell death inhibitor p35 did not block the cell death induced by DMp53 (Miller, *J Cell Physiol* (1997) 173(2):178-182; Ohtsubo *et al.*, *Nippon Rinsho* (1996) 54(7):1907-1911). However, DMp53-induced apoptosis and the rough-eye phenotype in gl-DMp53 flies could be suppressed by co-expression of the human cyclin-dependent-kinase inhibitor p21. Because p21 overexpression blocks cells in the G1 phase of the cell cycle, this finding suggests that transit through the cell cycle sensitizes cells to DMp53-induced apoptosis. A similar effect of p21 overexpression on human p53-induced apoptosis has been described.

p53 involvement in the cell cycle

In addition to its ability to affect cell death pathways, mammalian p53 can induce cell cycle arrest at the G1 and G2/M checkpoints. In the *Drosophila* eye disc, the second mitotic wave is a synchronous, final wave of cell division posterior to the morphogenetic furrow. This unique aspect of development provides a means to assay for similar effects of DMp53 on the cell. The transition of cells from G1 to S phase can be detected by BrdU incorporation. Eye discs dissected from wild-type third instar larvae displayed a tight band of BrdU-staining cells corresponding to DNA replication in the cells of the second mitotic wave. This transition from G1 to S phase was unaffected by DMp53 overexpression from the gl-DMp53 transgene. In contrast, expression of human p21 or a *Drosophila* homologue, dacapo (de Noij *et al.*, *Cell* (1996) 87(7):1237-1247; Lane *et al.*, *Cell* (1996) 87(7):1225-1235), under control of *glass*-responsive enhancer elements completely blocked DNA

replication in the second mitotic wave. In mammalian cells, p53 induces a cell cycle block in G1 through transcriptional activation of the p21 gene. These results suggest that this function is not conserved in DMp53.

In wild-type eye discs, the second mitotic wave typically forms a distinct band of cells that stain with an anti-phospho-histone antibody. In *gl*-DMp53 larval eye discs, this band of cells was significantly broader and more diffuse, suggesting that DMp53 alters the entry into and/or duration of M phase.

p53 response to DNA damage

The following experiments were performed to determine whether loss of DMp53 function affected apoptosis or cell cycle arrest in response to DNA damage.

In order to examine the phenotype of tissues deficient in DMp53 function, dominant-negative alleles of DMp53 were generated. These mutations are analogous to the R175H (R155H in DMp53) and H179N (H159N in DMp53) mutations in human p53. These mutations in human p53 act as dominant-negative alleles, presumably because they cannot bind DNA but retain a functional tetramerization domain. Co-expression of DMp53 R155H with wild-type DMp53 suppressed the rough eye phenotype that normally results from wild type DMp53 overexpression, confirming that this mutant acts as a dominant-negative allele *in vivo*. Unlike wild type DMp53, overexpression of DMp53 R155H or H159N using the *glass* enhancer did not produce a visible phenotype, although subtle alterations in the bristles of the eye were revealed by scanning electron microscopy.

In mammalian systems, p53-induced apoptosis plays a crucial role in preventing the propagation of damaged DNA. DNA damage also leads to apoptosis in *Drosophila*. To determine if this response requires the action of DMp53, dominant-negative DMp53 was expressed in the posterior compartment of the wing disc. Following X-irradiation, wing discs were dissected. TUNEL staining revealed apoptotic cells and anti-DMp53 antibody revealed the expression pattern of dominant-negative DMp53. Four hours after X-irradiation, wild type third instar larval wing discs showed widespread apoptosis. When the dominant-negative allele of DMp53 was expressed in the posterior compartment of the wing disc, apoptosis was blocked in the cells expressing DMp53. Thus, induction of apoptosis following X-irradiation requires the function of DMp53. This pro-apoptotic role for DMp53 appears to be limited to a specific response to cellular damage, because developmentally programmed cell death in the eye and other tissues is unaffected by expression of either dominant-negative DMp53 allele. The requirement for DMp53 in the

apoptotic response to X-irradiation suggests that DMp53 may be activated by DNA damage. In mammals, p53 is activated primarily by stabilization of p53 protein.

Although DMp53 function is required for X-ray induced apoptosis, it does not appear to be necessary for the cell cycle arrest induced by the same dose of irradiation. In the absence of irradiation, a random pattern of mitosis was observed in 3rd instar wing discs of *Drosophila*. Upon irradiation, a cell cycle block occurred in wild-type discs as evidenced by a significant decrease in anti-phospho-histone staining. The cell cycle block was unaffected by expression of dominant-negative DMp53 in the posterior of the wing disc. Several time points after X-irradiation were examined and all gave similar results, suggesting that both the onset and maintenance of the X-ray induced cell cycle arrest is independent of DMp53.

p53 in normal development

Similar to p53 in mice, DMp53 does not appear to be required for development because widespread expression of dominant-negative DMp53 in *Drosophila* had no significant effects on appearance, viability, or fertility. Interestingly, *in situ* hybridization of developing embryos revealed widespread early embryonic expression that became restricted to primordial germ cells in later embryonic stages. This expression pattern may indicate a crucial role for DMp53 in protecting the germ line, similar to the proposed role of mammalian p53 in protection against teratogens.

20

Example 13: p53 RNAi experiments in cell culture

Stable *Drosophila* S2 cell lines expressing hemagglutinin epitope (HA) tagged p53, or vector control under the inducible metallothionein promoter were produced by transfection using pMT/V5-His (Invitrogen, Carlsbad, CA). Induction of DMp53 expression by addition of copper to the medium resulted in cell death via apoptosis. Apoptosis was measured by three different methods: a cell proliferation assay; FACS analysis of the cell population in which dead cells were detected by their contracted nuclei; and a DNA ladder assay. The ability to use RNAi in S2 cell lines allowed p53 regulation and function to be explored using this inducible cell-based p53 expression system.

Preparation of the dsRNA template: PCR primers containing an upstream T7 RNA polymerase binding site and downstream DMp53 gene sequences were designed such that sequences extending from nucleotides 128 to 1138 of the DMp53 cDNA sequence (SEQ ID NO:1) could be amplified in a manner that would allow the generation of a

DMp53-derived dsRNA. PCR reactions were performed using EXPAND High Fidelity (Boehringer Mannheim, Indianapolis, IN) and the products were then purified.

DMp53 RNA was generated from the PCR template using the Promega Large Scale RNA Production System (Madison, WI) following manufacturer's protocols. Ethanol
5 precipitation of RNA was performed and the RNA was annealed by a first incubation at 68°C for 10 min, followed by a second incubation at 37°C for 30 min. The resulting dsRNA was stored at -80°C.

RNAi experiment in tissue culture: RNAi was performed essentially as described previously (<http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html>). On
10 day 1, cultures of Drosophila S2 cells were obtained that expressed pMT-HA-DMp53 expression plasmid and either 15 µg of DMp53 dsRNA or no RNA was added to the medium. On the second day, CuSO₄ was added to final concentrations of either 0, 7, 70 or 700 µM to all cultures. On the fourth day, an alamarBlue (Alamar Biosciences Inc.,
15 culture, by measuring fluorescence at 590 nm.

At 7µM CuSO₄, there was no change in cell number from 0 µM CuSO₄ for RNAi treated or untreated cells. At 70 µM CuSO₄, there was no change in cell number from 0 µM CuSO₄ for the RNAi-treated category. However, the number of cells that were not treated with RNAi dropped by 30%. At 700 µM CuSO₄, the number of cells that were treated with
20 RNAi dropped by 30% (as compared with 0µM CuSO₄), while the number of cells that were not treated with RNAi dropped by 70%.

These experiments showed that p53 dsRNA rescued at least 70% of the cells in the p53 inducible category, since some cell loss might be attributable to copper toxicity. Results of these experiments demonstrate that DMp53 dsRNA rescues cells from apoptosis
25 caused by inducing DMp53 overexpression. Thus, this experimental cell-based system represents a defined and unique way to study the mechanisms of p53 function and regulation.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:
 - 5 (a) a nucleic acid sequence that encodes a polypeptide comprising at least 7 contiguous amino acids of any one of SEQ ID NOs 4, 6, 8, and 10;
 - (b) a nucleic acid sequence that encodes a polypeptide comprising at least 7 contiguous amino acids of SEQ ID NO:2, wherein the isolated nucleic acid molecule is less than 15kb in size;
 - 10 (c) a nucleic acid sequence that encodes a polypeptide comprising at least 9 contiguous amino acids that share 100% sequence similarity with 9 contiguous amino acids of any one of SEQ ID NOs 4, 6, 8, and 10;
 - (d) a nucleic acid sequence that encodes a polypeptide comprising at least 9 contiguous amino acids that share 100% sequence similarity with 9 contiguous
15 amino acids of SEQ ID NO 2; wherein the isolated nucleic acid molecule is less than 15kb in size;
 - (e) at least 20 contiguous nucleotides of any of nucleotides 1-111 of SEQ ID NO:1, 1-120 of SEQ ID NO:3, 1-93 of SEQ ID NO:5, and 1-1225 of SEQ ID NO:18;
 - (f) a nucleic acid sequence that encodes a polypeptide comprising an amino acid
20 sequence having at least 80% sequence similarity with a sequence selected from the group consisting of SEQ ID NO:20 and SEQ ID NO:22; and
 - (g) the complement of the nucleic acid of any of (a)-(f).
2. The isolated nucleic acid molecule of Claim 1 that is RNA.
25
3. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence has at least 50% sequence identity with a sequence selected from the group consisting of any of SEQ ID NOs:1, 3, 5, 7, 9, 18, 19 and 21.
- 30 4. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a polypeptide comprising an amino acid sequence selected from the group consisting of: RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, KVCTCPGRD, FXCKNSC and FXCQNSC, wherein X is any amino acid.

5. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes at least 17 contiguous amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10.
- 5 6. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a polypeptide comprising at least 19 amino acids that share 100% sequence similarity with 19 amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10.
7. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence
10 encodes a polypeptide having at least 50% sequence identity with any of SEQ ID NOs 2, 4, 6, 8, and 10.
8. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes at least one p53 domain selected from the group consisting of an activation
15 domain, a DNA binding domain, a linker domain, an oligomerization domain, and a basic regulatory domain.
9. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a constitutively active p53.
- 20 10. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a dominant negative p53.
11. A vector comprising the nucleic acid molecule of Claim 1.
- 25 12. A host cell comprising the vector of Claim 11.
13. A process for producing a p53 polypeptide comprising culturing the host cell of Claim 8 under conditions suitable for expression of the p53 polypeptide and
30 recovering the polypeptide.
14. A purified polypeptide comprising an amino acid sequence selected from the group consisting of:
 - a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10;

- b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
- c) at least 10 contiguous amino acids of a sequence selected from the group consisting of SEQ ID NO:20 and SEQ ID NO:22.

5

15. The purified polypeptide of Claim 14 wherein the amino acid sequence is selected from the group consisting of RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, KVCTCPGRD, FXCKNSC and FXCQNSC, wherein X is any amino acid.

10

16. The purified polypeptide of Claim 14 wherein the amino acid sequence has at least 50% sequence similarity with a sequence selected from the group consisting of SEQ ID NOs 2, 4, 6, 8, and 10.

15

17. A method for detecting a candidate compound or molecule that modulates p53 activity said method comprising contacting a p53 polypeptide, or a nucleic acid encoding the p53 polypeptide, with one or more candidate compounds or molecules, and detecting any interaction between the candidate compound or molecule and the p53 polypeptide or nucleic acid; wherein the p53 polypeptide comprises an amino acid sequence selected from the group consisting of:

20

- a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
- b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10.

25

18. The method of Claim 17 wherein the candidate compound or molecule is a putative pharmaceutical agent.

30

19. The method of Claim 17 wherein the contacting comprises administering the candidate compound or molecule to cultured host cells that have been genetically engineered to express the p53 protein.

20. The method of Claim 17 wherein the contacting comprises administering the candidate compound or molecule to an insect has been genetically engineered to express the p53 protein.
- 5 21. The method of Claim 20 wherein the candidate compound is a putative pesticide.
22. A first insect that has been genetically modified to express or mis-express a p53 protein, or the progeny of the insect that has inherited the p53 protein expression or mis-expression, wherein the p53 protein comprises an amino acid sequence selected from the group consisting of:
- 10 a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10.
- 15 23. The insect of Claim 22 wherein said insect is *Drosophila* that has been genetically modified to express a dominant negative p53 having a mutation selected from the group consisting of R155H, H159N, and R266T.
- 20 24. A method for studying p53 activity comprising detecting the phenotype caused by the expression or mis-expression of the p53 protein in the first insect of Claim 22.
- 25 25. The method of Claim 24 additionally comprising observing a second insect having the same genetic modification as the first insect which causes the expression or mis-expression of the p53 protein, and wherein the second animal additionally comprises a mutation in a gene of interest, wherein differences, if any, between the phenotype of the first animal and the phenotype of the second animal identifies the gene of interest as capable of modifying the function of the gene encoding the p53 protein.
- 30 26. The method of Claim 24 additionally comprising administering one or more candidate compounds or molecules to the insect or its progeny and observing any changes in p53 activity of the insect or its progeny.

27. A method of modulating p53 activity comprising contacting an insect cell with the isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule is dsRNA derived from a coding region of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, and 9.

5

28. The method of Claim 27 wherein cultured insect cells are contacted with the dsRNA and apoptosis of the cultured cells is assayed.

Fig. 1A

Human_p53 DEEPQSDPSVEPPLSCQETFSDDMKLDPENNV-----LSP--LPSQAMD
Xeno_p53 ME-PSSETQMDPPLSQETFEEDMSLPP-D-----PLQTVT
Squid_p53 MSQGTG-----PNSQETPNLWDSDEQVTANEYTOHERGVGYEHEAEPDQTSLEISA
CPB_p53 MS-QQSD-----FLPPDVQNFLLAEMEGD-----NMDNLN
Trib_p53A MS-QQS-----QFSIIPDVDFKEDH-----GLKDDV
Trib_p53B -----V
Helio_p53 -----V
Dros_p53 MYISQP-----MSWHKEETDSEDDSTEDVIK-----EDIPKTV
consensus m qs et l le 1

Human_p53 DLMLEFDDI---EQWFTEDPGPDEAPRMPEAAAPRV-----APAPAAPTPAAPAPA[SW
Xeno_p53 CRLDNLS-----EPDYPLAADMT-----VLQEGLMGNAPVTVT---
Squid_p53 YRIAQDPYGRSESYDLLNPIINQIPAPMPIADTQNNPLVNHCPEYEDMPVSSTPYSPDH
CPB_p53 FFKDEET-----LNDLNSNILNG-----SIVANDDSKMVHLIFPG-
Trib_p53A GRIMHEN-----NVHLVNDDE-----EKEYSNEANYTESIFP-
Trib_p53B RQYKPED-----FSHTFHPIICSIQLEDKFNINQSSYLSAPIFEP-
Helio_p53 -----
Dros_p53 EVSGSELT-----TEMAFLQGLNSGN-----LMQFSQQSVLRMMLODI
consensus m p a t v p

Human_p53 PL--SSSVPSQKTQCSGCEIRGLFLH-SGTAKSVTCTTSPALNNKMFQOLAKTCPPVQLWVD
Xeno_p53 ---SCAVPSDDDYACKYGLQDFDQ-NGTAKSVTCTTSPALNNKMFQOLAKTCPLLVRE
Squid_p53 VQSPQSPVSNIXKPYCEVTEMSFAQPSKETKSTTWYSEKDDKLVVRMATTCPVRKTA
CPB_p53 ---VQTSVPSNDEYDCPESEVDVHP---TVAKNSVVTSTANKAVMTGSPFVDERVS
Trib_p53A ---DQPTNLGTEETPCPFNSVLISPP---NEQKSPWEYSEKGNKFIIGINVKFPPVAFSVQ
Trib_p53B ---SEPLELCNTEYPCPLNEVEFVDP---NVLKNPMEYSPILANKIVIDMKHKPPIINESVK
Helio_p53 QIQANTLPKLENHNIGGTCESMVLDE---PPKSLIMYSIPINELVIRNKAENVDVQFK
Dros_p53 q svpsq dy g y f l v k tw ys lmkmyv ma fpv f v
consensus

Human_p53 STPP-PGTRVRAMAIYKQSQHMTVEVVRCPHHERCSD-----SDGLAPPQHLIRVEGNLR
Xeno_p53 SPPP-RGSIIRATAYKKSEHVAEIVWKRCPHHERSVEP-----GEDAAPPSHLRVEGNLQ
Squid_p53 RPPP-SGCQIRAMPIYMKPEHVOEVVKRCPNHATAKEH-----NEKHPAPLHVRCEHKL-
CPB_p53 HRPP-NPLFIRSTPVYSAPQAFQECVYRCINHEFSHKEH-DGDLKEHIRPFIIRCANQYA
Trib_p53A NRQNLPLYIRATPVFSQTHFQDLVHRCVGVHRHPDQDS-NKGVAPHIFQHIIRCTNDNA
Trib_p53B KADEERRLEVRVMPMEEDRYVQELVHRCICHEQLDPT-NHNVSEMVACHIRCDNNNA
Helio_p53 YQKA-PHMFVRSVTVFSDQTAEKRRVERCVQHFHESSTSGIQTEIAKNVLHSSREIGTQG
Dros_p53 SKMEIQP[ENLRVFLCFSDN--VSAPVVRCONHLSVEPLT---ANNAKMRESLLRSENPNPS
consensus pp lfvratpvys hvqevv rclnHe d s d i hiircennna

Human_p53 VEYLD[DRNT---FRHSVVVPYEP-----FEVGSDDCTTIHYNYMCNNSCMG-GMNRRLPILTH
Xeno_p53 AYMEDVNS---GRHSVCVPYEG-----POVGTECTTVLYNYMCNNSCMG-GMNRRLPILTH
Squid_p53 AKYHEDKYS---GRQSVLIPHEM-----POAGSEMVVNLYQFMCLGSCVG-GMNRRLPILV
CPB_p53 AYLGDKSKN---ERLSVVPFPGI---PQGTESVREIFEVCKNSCPSPGMNRRAVEIH
Trib_p53A LYFGDKNTG---TRLNIVLPLAH---PQGEDVVKEFFQEVCKNSCPL-GMNRRLPIDVV
Trib_p53B QYFGDKNAG---KRLS-----
Helio_p53 VYFCGKVDN---ADSWYSVLVEF---MRTSESCHAYVQFSCKNSCAT-GINRRRAIAI
Dros_p53 VYCGNAQCGKISERFVSVPFLNMSRVSRTGLRQTLAKFVQNSCHG---RRKETSIV
consensus vyygdk i svvvp e pq gse t ynfmc nscmg gmnrip i i

Fig. 1B

```

Human_p53  ITLEDSSGNIIGRMFSFEVRVVCACPGDRDRRTTEE-NLRKKG-----EPHHELPPGSTRKRALP
Xeno_p53    ITLETPOGLIIGRRRCFEVRVVCACPGDRDRRTTEED-NYTKK-----RGLKBSG--KRELA
Squid_p53   FTLEK-DNQVLGRRAVEVRVICACPGDRDKADEKASLVSK-----PPSPKNGFPQRSLV
CPB_p53     FTLEDNQGTIVYGRKTNVRIICSPGRDKRDKERDNTANT-----NLPHG-KRKKME
Trib_p53A   FTLEDNKGEVFCRRLLVGVVVCSPGRDKDKZEK-DMESA-----VPRRKKRKLG
Trib_p53B   FTLEDAMGNIHGRQKVGAH-----
Helio_p53   ECLEKACGDIVGQHVIHVKICTCEPKRDRIQDERQLNSKKRKSVPAAAEDEPSKVRRCIA
Dros_p53    ftled  g llgr v vivc cp rdx eek  k p g kr l
consensus  -----

Human_p53  NNTSSSP-QPKK---KPLDGEYFTLQIRGRERFERFRELNEAELKDAQAG-----
Xeno_p53   HPPSEPPLPKKRLVDDDEEIFTLRIKGRSRYEMIKKLNDAELQESLDQ-----
Squid_p53  LTNDITKITPKK---RKIDDECFTLKVGRGNEYEILCKLRDIMELAAARIPEAERLLYKQE
CPB_p53    KPSKKPMQTQAE---NDTKFTLTIPLVGRHNEQNVLYKCHDMAGEILRN-----
Trib_p53A  NDERRVVPQSS---DNKIFALNIHIPCKKNYLOALMCQDMLANEILKKQ-----
Trib_p53B  -----
Helio_p53  -----
Dros_p53   IKTEDTESNDSRDCDDSAAEWNVSRTPDCDYRLAITCPNKKEWTLQSIEGMK-----
consensus  k e tl i gr f m kl e l d l

Human_p53  -KEPGC---SRAHSHLKSCKKQ-----STSRHKKLMFKTEGPDSD-----
Xeno_p53   -KVTIK---CRKCRDEINPKKG-----KKLLVKDEQPDSE-----
Squid_p53  RQAPICRLTSLPSSSSNGSQDGSRSSTAFSTSDSSQVNSSQNNTQMVNGQVHEEETPVT
CPB_p53    ---IG---NGTEGPKIALN-----KINTLIRESE-----
Trib_p53A  -EQC---GD-DSADKNCYN-----EITVLLNGTAAFD-----
Trib_p53B  -----
Helio_p53  -----
Dros_p53   -EAAAEVLNPNQENLRHAN-----KLLSILKKRAYELP-----
consensus  g k 1mv d

Human_p53  -----
Xeno_p53   -----
Squid_p53  KCEPTIAQWLTKLGLQAYIDNFQOKGLHNMFPOLDEFTLEDLQSMRIGTGHNRNKIWK
CPB_p53    -----
Trib_p53A  -----
Trib_p53B  -----
Helio_p53  -----
Dros_p53   -----
consensus  -----

Human_p53  -----
Xeno_p53   -----
Squid_p53  LLDYRRLSSGTESQALQHAASNASTLSVGSQNSYCPGFYEVTYTKHTISYL
CPB_p53    -----
Trib_p53A  -----
Trib_p53B  -----
Helio_p53  -----
Dros_p53   -----
consensus  -----

```


SEQUENCE LISTING

<110> EXELIXIS, INC

<120> Insect p53 Tumor Suppressor Genes and Proteins

<130> Insect p53 sequences

<140> EX00-015

<141> 2000-03-13

<150> EX99-001

<151> 1999-03-16

<160> 22

<170> PatentIn Ver. 2.1

<210> 1

<211> 1573

<212> DNA

<213> Drosophila melanogaster

<400> 1

```

aaaatccaaa tagtcggtgg ccactacgat tctgtagttt tttgtagcg aatttttaat 60
atttagcctc cttccccaac aagatcgctt gatcagatat agccgactaa gatgtatata 120
tcacagccaa tgtcgtggca caaagaaagc actgattccg aggatgactc cacggaggtc 180
gatatcaagg aggatattcc gaaaacgggtg gaggtatcgg gatcggaatt gaccacggaa 240
cccatggcct tcttcgaggg attaaactcc gggaaatctga tgcagttcag ccagcaatcc 300
gtgctgcgcg aaatgatgct gcaggacatt cagatccagg cgaacacgct gccaagcta 360
gagaatcaca acatcggtgg ttattgcttc agcatgggtc tggatgagcc gccaagtct 420
ctttggatgt actcgattcc gctgaacaag ctctacatcc ggatgaacaa ggccttcaac 480
gtggacgttc agttcaagtc taaaatgccc atccaaccac ttaatttgcg tgtgttcctt 540
tgcttctcca atgatgtgag tgctcccggtg gtcgctgtc aaaatcacct tagcgttgag 600
cctttgacgg ccaataacgc aaaaatgcgc gagagcttgc tgcgcagcga gaatcccaac 660
agtgtatatt gtggaaatgc tcagggcaag ggaatttcg agcgtttttc cgtttagtagt 720
cccctgaaca tgagccggtc tgtaaccgcg agtgggctca cgcgccagac cctggccttc 780
aagttcgtct gccaaaactc gtgtatcggg cgaaaagaaa cttccttagt cttctgcctg 840
gagaaagcat gcggcgatat cgtgggacag catgctatac atgttaaaat atgtacgtgc 900
cccaagcggg atcgcatcca agacgaacgc cagctcaata gcaagaagcg caagtccgtg 960
ccggaagccg ccgaagaaga tgagccgtcc aaggcgcgctc ggtgcattgc tataaagacg 1020
gaggacacgg agagcaatga tagccgagac tgcgacgact ccgccgcaga gtggaacgtg 1080
tcgcggaacac cggatggcga ttaccgtctg gctattacgt gcccataa ggaatggctg 1140
ctgcagagca tcgagggcat gattaaggag gcggcggtc aagtcctgcg caatcccaac 1200
caagagaatc tacgtcgcca tgccaacaaa ttgctgagcc ttaagaaacg tgcctacgag 1260
ctgccatgac ttctgatctg gtcgacaatc tcccaggat cagatacctt tgaatcgtgt 1320
tgcattctgtg gggataacta catagctatt agtatcttaa gtttgtatta gtccttgttc 1380
gtaaggcggtt taacggtgat attccctttt tggcatgttc gatggccgaa aagaaaacat 1440

```

```

ttttatattt ttgatagtat actgttggtta actgcagttc tatgtgacta cgtaactttt 1500
gtctaccaca acaaacatac tctgtacaaa aaagccaaaa gtgaattttat taaagagttg 1560
tcatattttg caa 1573

```

<210> 2

<211> 385

<212> PRT

<213> Drosophila melanogaster

<400> 2

Met Tyr Ile Ser Gln Pro Met Ser Trp His Lys Glu Ser Thr Asp Ser
1 5 10 15

Glu Asp Asp Ser Thr Glu Val Asp Ile Lys Glu Asp Ile Pro Lys Thr
20 25 30

Val	Glu	Val	Ser	Gly	Ser	Glu	Leu	Thr	Thr	Glu	Pro	Met	Ala	Phe	Leu
		35					40					45			

Gln Gly Leu Asn Ser Gly Asn Leu Met Gln Phe Ser Gln Gln Ser Val
50 55 60

Leu Arg Glu Met Met Leu Gln Asp Ile Gln Ile Gln Ala Asn Thr Leu
65 70 75 80

Pro Lys Leu Glu Asn His Asn Ile Gly Gly Tyr Cys Phe Ser Met Val
85 90 95

Leu Asp Glu Pro Pro Lys Ser Leu Trp Met Tyr Ser Ile Pro Leu Asn
100 105 110

Lys Leu Tyr Ile Arg Met Asn Lys Ala Phe Asn Val Asp Val Gln Phe
115 120 125

Lys Ser Lys Met Pro Ile Gln Pro Leu Asn Leu Arg Val Phe Leu Cys
130 135 140

Phe Ser Asn Asp Val Ser Ala Pro Val Val Arg Cys Gln Asn His Leu
145 150 155 160

Ser Val Glu Pro Leu Thr Ala Asn Asn Ala Lys Met Arg Glu Ser Leu
165 170 175

Leu Arg Ser Glu Asn Pro Asn Ser Val Tyr Cys Gly Asn Ala Gln Gly
180 185 190

Lys Gly Ile Ser Glu Arg Phe Ser Val Val Val Pro Leu Asn Met Ser

195 200 205
 Arg Ser Val Thr Arg Ser Gly Leu Thr Arg Gln Thr Leu Ala Phe Lys
 210 215 220
 Phe Val Cys Gln Asn Ser Cys Ile Gly Arg Lys Glu Thr Ser Leu Val
 225 230 235 240
 Phe Cys Leu Glu Lys Ala Cys Gly Asp Ile Val Gly Gln His Val Ile
 245 250 255
 His Val Lys Ile Cys Thr Cys Pro Lys Arg Asp Arg Ile Gln Asp Glu
 260 265 270
 Arg Gln Leu Asn Ser Lys Lys Arg Lys Ser Val Pro Glu Ala Ala Glu
 275 280 285
 Glu Asp Glu Pro Ser Lys Val Arg Arg Cys Ile Ala Ile Lys Thr Glu
 290 295 300
 Asp Thr Glu Ser Asn Asp Ser Arg Asp Cys Asp Asp Ser Ala Ala Glu
 305 310 315 320
 Trp Asn Val Ser Arg Thr Pro Asp Gly Asp Tyr Arg Leu Ala Ile Thr
 325 330 335
 Cys Pro Asn Lys Glu Trp Leu Leu Gln Ser Ile Glu Gly Met Ile Lys
 340 345 350
 Glu Ala Ala Ala Glu Val Leu Arg Asn Pro Asn Gln Glu Asn Leu Arg
 355 360 365
 Arg His Ala Asn Lys Leu Leu Ser Leu Lys Lys Arg Ala Tyr Glu Leu
 370 375 380

Pro
385

<210> 3

<211> 2600

<212> DNA

<213> Leptinotarsa decemlineata

<400> 3

gtgttttagtt attgttcggg ggctgttttt ttaattaaaa atttcacggg taaatctttg 60
 ttgtcttttc tttttctaatt tgtatcagaa tagctttttt aactgtgaaa accggaaggg 120
 atgtcttttc agtcagactt ttacctcca gatgttcaaa atttctctt gccagaaatg 180

```

gaaggggaca atatggataa tctaaacttt ttcaaggacg aaccaacttt gaatgattta 240
aattattcaa acatcctaaa tggatcaata gttgctaata atgattcaaa gatggttcat 300
cttattttttc cgggagtaca aacaagtgtc ccatcaaata atgaatacga tgggtccatat 360
gaatttgaag tagatgttca tccactgtg gcaaaaaatt cgtgggtgta ctctaccacc 420
ctgaataaag tttatatgac aatgggcagt ccatttcttg tagatttcag agtatcacat 480
cgacccccga acccattatt catcaggagc actcccgttt acagtgtctc ccaatttgct 540
caagaatgtg tttaccggtg cctaaacat gaattctctc ataaagagtc tgatggagat 600
ctcaaggaac acattcgccc tcatatcata agatgtgcca atcagtatgc tgcttactta 660
gggtgacaagt ctaaaaatga acgtctcagc gttgtcatac cattcggtat ccgcgagacg 720
gggtactgaaa gtgttagaga aatttttcgaa tttgtttgca aaaattcttg cccaagtcct 780
ggaatgaata gaagagctgt ggaaataata ttcacttttg aggataatca aggaactatc 840
tatggacgca aaacattaaa tgtgagaata tgctcttgct caaacgtga taaagagaaa 900
gatgaaaagg ataacactgc caacactaat ctgccgcag gcaaaaagag aaaaatggag 960
aagccatcaa agaaacccat gcagacacag gcagaaaatg ataccaaaga gtttactctg 1020
accataccgc tgggtgggtcg acataatgaa caaatgtgt tgaagtattg ccatgatttg 1080
atggccgggg aaatcctgcg aaatatcggc aatggtagtg aagggccgta caaatagct 1140
ttaaacaaaa taaacacgtt gatacgtgaa agttccgagt gaccttatca attctatgta 1200
tatttcttat acaattccat tttcatattt ccatttgata ataagaaaca ttttagcacc 1260
ttttaatcct aactgcagg gaagtcaata tttctttagt tttttgcatg atattgtttg 1320
ttataacatt ttttttttca acaacagggt acttgatttt tgtaagggtat ctcatatttt 1380
atgtttaaga cctaaaacac gaaacaaaa acatgaatgg tcattgaatt tggctcgata 1440
atcaatccaa tgttctttta agtaatatcg acctgttcac aacttttgtg atgcactgaa 1500
tggcttttta ttattattat ttttcagcat tgtacatcat acttgcatag tttcagtttt 1560
aaatttttca aatgtttcat ttattttcat tcttacacct gaacttggat tttggacaca 1620
tggctttcac aatgttctat cacgaacagt atgataagcc aaagtaagag ttgataatag 1680
ttcatattaa tatctattgt aacaccgact attgttatat aaatagtcgt ttttttgta 1740
cttttcttgc tttattttat acacttgagt caagtgtagt cagtacattg actatgctgg 1800
aaaacctgtt ttgagtttat ttttacttac attcagttct catcattaga aattgtttat 1860
tttttgtgtg caatattttac gaaaaatggt gcaatactat aataggaaca ttaataaagt 1920
aacttgaaag catagagggtg gtgaattttg tttttgatca actttttgaa atttatgctg 1980
cattctataa gccagttttt tttgataaat tcaaaattca cgaataggta tcaacctgat 2040
tgcagtctta ttctatgttt gtcctaaagc aggtctctat aaaacttctc taaaagttgt 2100
gcagagcaaa taacaaataa ttttttaatg gattatatca attcatgaac tggtttaatt 2160
gaaagagtag attattctat tgggttcaca aaaatataaa taatgtgtta ctatctggat 2220
catttgtttt tttttcattg agctatatat tgtcattgta ttgttgaact ttccctaaat 2280
cccagtcca tagtcgacga tcggtctcgc tcccatccat caattattcg aaatctcatt 2340
tattttaaag actgaggacg ggggtgggact gtcagtgtat ctgtttaatg agaaccatct 2400
tgtactagga ttgatatgtg aatctatgag taggtgcatt ttatatata tatctttatg 2460
tttatttagt attattgtac aggttatgta ctctagtgga agaatacata acctaatat 2520
tatatatgtt cggttaataa caaatttttt acgtttttta aatatatttt ctaaatattc 2580
aacaacaaaa aaaaaaaaaa
2600

```

<210> 4

<211> 354

<212> PRT

<213> Leptinotarsa decemlineata

<400> 4

Met Ser Ser Gln Ser Asp Phe Leu Pro Pro Asp Val Gln Asn Phe Leu
 1 5 10 15
 Leu Ala Glu Met Glu Gly Asp Asn Met Asp Asn Leu Asn Phe Phe Lys
 20 25 30
 Asp Glu Pro Thr Leu Asn Asp Leu Asn Tyr Ser Asn Ile Leu Asn Gly
 35 40 45
 Ser Ile Val Ala Asn Asp Asp Ser Lys Met Val His Leu Ile Phe Pro
 50 55 60
 Gly Val Gln Thr Ser Val Pro Ser Asn Asp Glu Tyr Asp Gly Pro Tyr
 65 70 75 80
 Glu Phe Glu Val Asp Val His Pro Thr Val Ala Lys Asn Ser Trp Val
 85 90 95
 Tyr Ser Thr Thr Leu Asn Lys Val Tyr Met Thr Met Gly Ser Pro Phe
 100 105 110
 Pro Val Asp Phe Arg Val Ser His Arg Pro Pro Asn Pro Leu Phe Ile
 115 120 125
 Arg Ser Thr Pro Val Tyr Ser Ala Pro Gln Phe Ala Gln Glu Cys Val
 130 135 140
 Tyr Arg Cys Leu Asn His Glu Phe Ser His Lys Glu Ser Asp Gly Asp
 145 150 155 160
 Leu Lys Glu His Ile Arg Pro His Ile Ile Arg Cys Ala Asn Gln Tyr
 165 170 175
 Ala Ala Tyr Leu Gly Asp Lys Ser Lys Asn Glu Arg Leu Ser Val Val
 180 185 190
 Ile Pro Phe Gly Ile Pro Gln Thr Gly Thr Glu Ser Val Arg Glu Ile
 195 200 205
 Phe Glu Phe Val Cys Lys Asn Ser Cys Pro Ser Pro Gly Met Asn Arg
 210 215 220
 Arg Ala Val Glu Ile Ile Phe Thr Leu Glu Asp Asn Gln Gly Thr Ile
 225 230 235 240
 Tyr Gly Arg Lys Thr Leu Asn Val Arg Ile Cys Ser Cys Pro Lys Arg
 245 250 255

Asp Lys Glu Lys Asp Glu Lys Asp Asn Thr Ala Asn Thr Asn Leu Pro
 260 265 270

His Gly Lys Lys Arg Lys Met Glu Lys Pro Ser Lys Lys Pro Met Gln
 275 280 285

Thr Gln Ala Glu Asn Asp Thr Lys Glu Phe Thr Leu Thr Ile Pro Leu
 290 295 300

Val Gly Arg His Asn Glu Gln Asn Val Leu Lys Tyr Cys His Asp Leu
 305 310 315 320

Met Ala Gly Glu Ile Leu Arg Asn Ile Gly Asn Gly Thr Glu Gly Pro
 325 330 335

Tyr Lys Ile Ala Leu Asn Lys Ile Asn Thr Leu Ile Arg Glu Ser Ser
 340 345 350

Glu Trp

<210> 5

<211> 1291

<212> DNA

<213> Tribolium castaneum

<400> 5

acgcgtccgg ccaacttaac ctaaaaatgt gttttcgatg cctactagat ttaaaaacaa 60
 ttgattcaaa tcgtggattt ttattattta aatcatgagc caacaaagtc aatttttcgga 120
 catcattcct gatgttgata aatttttgga agatcatgga ctcaaggacg atgtgggaag 180
 aataatgcac gaaaacaacg tccatttagt aaatgacgac ggagaagaag aaaaatactc 240
 taatgaagcc aattacactg aatcaatttt ccccccgac cagcccacaa acctaggcac 300
 tgaggaatac ccaggccctt ttaattttct agtcctgatc agccccaacg agcaaaaatc 360
 gccctgggag tattcggaaa aactgaacaa aatattcatc ggcatcaacg tgaaattccc 420
 cgtggccttc tccgtgcaaa accgccccca gaacctgccc ctctacatcc gcgccacccc 480
 cgtgttcagc caaacgcagc acttccaaga cctgggtgcac cgctgcgtcg gccaccgcca 540
 ccccaagac cagtccaaca aaggcgtcgc ccccccatt ttccagcaca ttattagggtg 600
 caccaacgac aacgcccatt actttggcga taaaaacaca gggacgagac tcaacatcgt 660
 cctgcctttg gccaccccc aggtggggga ggacgtgggc aaggagtttt tccagtttgt 720
 gtgcaaaaac tccgtccctt tggggatgaa tcggcggcgc attgatgtcg ttttcacctt 780
 ggaggataat aagggggagg ttttcgggag gaggttggtg ggggtgaggg tgtgttcgtg 840
 tccgaagcgt gacaaggaca aggaggagaa ggacatggag agtgctgtgc ctccaaggag 900
 gaagaagagg aagttgggga atgatgagcg aagggttggt ccacagggga gctccgataa 960
 taaaatatgt gcgttaata ttcatattcc tggcaagaag aattattttac aagccctcaa 1020
 gatgtgtcaa gatatgctgg ctaatgaaat tttgaaaaaa caggaacaag gtggcgacga 1080
 ttctgctgat aagaactgtt ataatgagat aactgttctc ttgaacggca cggccgcctt 1140

tgattagttt atttctatat ttaattttat actttgtact tatgcaatat tccagtttac 1200
 ttttgaata tttttattaa taaatttcta cgttttaaaa aaaaaaaaaa aaaaaaaaaa 1260
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa a 1291

<210> 6

<211> 350

<212> PRT

<213> Tribolium castaneum

<400> 6

Met Ser Gln Gln Ser Gln Phe Ser Asp Ile Ile Pro Asp Val Asp Lys
 1 5 10 15

Phe Leu Glu Asp His Gly Leu Lys Asp Asp Val Gly Arg Ile Met His
 20 25 30

Glu Asn Asn Val His Leu Val Asn Asp Asp Gly Glu Glu Glu Lys Tyr
 35 40 45

Ser Asn Glu Ala Asn Tyr Thr Glu Ser Ile Phe Pro Pro Asp Gln Pro
 50 55 60

Thr Asn Leu Gly Thr Glu Glu Tyr Pro Gly Pro Phe Asn Phe Ser Val
 65 70 75 80

Leu Ile Ser Pro Asn Glu Gln Lys Ser Pro Trp Glu Tyr Ser Glu Lys
 85 90 95

Leu Asn Lys Ile Phe Ile Gly Ile Asn Val Lys Phe Pro Val Ala Phe
 100 105 110

Ser Val Gln Asn Arg Pro Gln Asn Leu Pro Leu Tyr Ile Arg Ala Thr
 115 120 125

Pro Val Phe Ser Gln Thr Gln His Phe Gln Asp Leu Val His Arg Cys
 130 135 140

Val Gly His Arg His Pro Gln Asp Gln Ser Asn Lys Gly Val Ala Pro
 145 150 155 160

His Ile Phe Gln His Ile Ile Arg Cys Thr Asn Asp Asn Ala Leu Tyr
 165 170 175

Phe Gly Asp Lys Asn Thr Gly Thr Arg Leu Asn Ile Val Leu Pro Leu
 180 185 190

Ala His Pro Gln Val Gly Glu Asp Val Val Lys Glu Phe Phe Gln Phe

195 200 205
 Val Cys Lys Asn Ser Cys Pro Leu Gly Met Asn Arg Arg Pro Ile Asp
 210 215 220
 Val Val Phe Thr Leu Glu Asp Asn Lys Gly Glu Val Phe Gly Arg Arg
 225 230 235 240
 Leu Val Gly Val Arg Val Cys Ser Cys Pro Lys Arg Asp Lys Asp Lys
 245 250 255
 Glu Glu Lys Asp Met Glu Ser Ala Val Pro Pro Arg Arg Lys Lys Arg
 260 265 270
 Lys Leu Gly Asn Asp Glu Arg Arg Val Val Pro Gln Gly Ser Ser Asp
 275 280 285
 Asn Lys Ile Phe Ala Leu Asn Ile His Ile Pro Gly Lys Lys Asn Tyr
 290 295 300
 Leu Gln Ala Leu Lys Met Cys Gln Asp Met Leu Ala Asn Glu Ile Leu
 305 310 315 320
 Lys Lys Gln Glu Gln Gly Gly Asp Asp Ser Ala Asp Lys Asn Cys Tyr
 325 330 335
 Asn Glu Ile Thr Val Leu Leu Asn Gly Thr Ala Ala Phe Asp
 340 345 350

<210> 7

<211> 508

<212> DNA

<213> Tribolium castaneum

<400> 7

gtacgacaat acaaaccgcc cgatttttcc cacactttcc acccaataat ttgctcaatt 60
 ttccagttgg aagacttcaa attcaacatc aaccaaagct cgtacctctc agccccatt 120
 ttcccccca gcgagccgct cgagctgtgc aacaccgagt acccgggccc cctcaacttc 180
 gaggtgtttg tggaccccaa cgtgctcaaa aacccttggg aatactcccc aattctcaac 240
 aaaattttaca tcgatatgaa acacaaattc ccgattaatt tcagcgtgaa gaaggccgat 300
 cctgagcgca ggctttttgt cagagttatg ccgatgtttg aggaagacag atatgtgcaa 360
 gaattggtgc ataggtgcat ctgtcacgaa caattgacag atccgaccaa tcacaacgtt 420
 tcggaaatgg tggctcagca catcattcgg tgtgataaca acaatgctca gtatttcggg 480
 gataagaacg ctgggaagag actgagta 508

<210> 8

<211> 169

<212> PRT

<213> Tribolium castaneum

<400> 8

Val Arg Gln Tyr Lys Pro Pro Asp Phe Ser His Thr Phe His Pro Ile
 1 5 10 15

Ile Cys Ser Ile Phe Gln Leu Glu Asp Phe Lys Phe Asn Ile Asn Gln
 20 25 30

Ser Ser Tyr Leu Ser Ala Pro Ile Phe Pro Pro Ser Glu Pro Leu Glu
 35 40 45

Leu Cys Asn Thr Glu Tyr Pro Gly Pro Leu Asn Phe Glu Val Phe Val
 50 55 60

Asp Pro Asn Val Leu Lys Asn Pro Trp Glu Tyr Ser Pro Ile Leu Asn
 65 70 75 80

Lys Ile Tyr Ile Asp Met Lys His Lys Phe Pro Ile Asn Phe Ser Val
 85 90 95

Lys Lys Ala Asp Pro Glu Arg Arg Leu Phe Val Arg Val Met Pro Met
 100 105 110

Phe Glu Glu Asp Arg Tyr Val Gln Glu Leu Val His Arg Cys Ile Cys
 115 120 125

His Glu Gln Leu Thr Asp Pro Thr Asn His Asn Val Ser Glu Met Val
 130 135 140

Ala Gln His Ile Ile Arg Cys Asp Asn Asn Asn Ala Gln Tyr Phe Gly
 145 150 155 160

Asp Lys Asn Ala Gly Lys Arg Leu Ser
 165

<210> 9

<211> 433

<212> DNA

<213> Heliothis virescens

<400> 9

gcacgagatg aagtgcact ttagcgtgca attcaactgg gactatcaga aggcgccgca 60
 tatgttcgtg cggctctaccg tcgtgttctc cgatgaaacg caggcggaga agcgggtcga 120
 acgatgtgtg cagcatttcc atgaaagctc cacttctgga atccaaacag aaattgccaa 180

aaacgtgctc cactcgtccc gggagatcgg taccagggc gtgtactact gcggaaggt 240
 ggacatggca gactcgtggt actcagtgtt ggtggagttt atgaggacca gtcggagtc 300
 ctgctcccat gcgtaccagt tctcctgcaa gaactcttgc gcaaccggca ttaataggcg 360
 ggctattgcc attattttta cgctggaaga tgctatgggc aacatccacg gccgtcagaa 420
 agtaggggag agg 433

<210> 10

<211> 144

<212> PRT

<213> *Heliothis virescens*

<400> 10

His Glu Met Lys Cys Asn Phe Ser Val Gln Phe Asn Trp Asp Tyr Gln
 1 5 10 15

Lys Ala Pro His Met Phe Val Arg Ser Thr Val Val Phe Ser Asp Glu
 20 25 30

Thr Gln Ala Glu Lys Arg Val Glu Arg Cys Val Gln His Phe His Glu
 35 40 45

Ser Ser Thr Ser Gly Ile Gln Thr Glu Ile Ala Lys Asn Val Leu His
 50 55 60

Ser Ser Arg Glu Ile Gly Thr Gln Gly Val Tyr Tyr Cys Gly Lys Val
 65 70 75 80

Asp Met Ala Asp Ser Trp Tyr Ser Val Leu Val Glu Phe Met Arg Thr
 85 90 95

Ser Ser Glu Ser Cys Ser His Ala Tyr Gln Phe Ser Cys Lys Asn Ser
 100 105 110

Cys Ala Thr Gly Ile Asn Arg Arg Ala Ile Ala Ile Ile Phe Thr Leu
 115 120 125

Glu Asp Ala Met Gly Asn Ile His Gly Arg Gln Lys Val Gly Ala Arg
 130 135 140

<210> 11

<211> 26

<212> DNA

<213> *Drosophila melanogaster*

<400> 11

ccatgctgaa gcaataacca ccgatg

26

<210> 12

<211> 30

<212> DNA

<213> Drosophila melanogaster

<400> 12

ggaacacacg caaattaagt gggttgatgg

30

<210> 13

<211> 23

<212> DNA

<213> Drosophila melanogaster

<400> 13

tgattttgac agcggaccac ggg

23

<210> 14

<211> 28

<212> DNA

<213> Drosophila melanogaster

<400> 14

ggaagtttct tttcgcccca tacacgag

28

<210> 15

<211> 27

<212> DNA

<213> Drosophila melanogaster

<400> 15

ggcaciaaga aagcactgat tccgagg

27

<210> 16

<211> 28

<212> DNA

<213> Drosophila melanogaster

<400> 16

ggaatctgat gcagttcagc cagcaatc

28

<210> 17
 <211> 23
 <212> DNA
 <213> *Drosophila melanogaster*

<400> 17
 ggatcgcatc caagacgaac gcc

23

<210> 18
 <211> 27425
 <212> DNA
 <213> *Drosophila melanogaster*

<400> 18
 tagccactcg ctagtttata gttcaagggt aacatacgt agagttttgt ggcaactggac 60
 tggaaatagg ctgctagtc tttgtgttcg gccatagcgt taaaaattta agccaacgcc 120
 agtcgtcctg cgcccatggt gctgcaacat tctggcttcg tgtcatgcc ctgaatgttt 180
 cacattattt aacccccctt attttttttt tttgtgtggc actggccaaa ggtccaaagg 240
 ggcgacatgc tgcagggggc tggcctgcag ctgcttgcaa cgggcaatta ttgcgcagtt 300
 attgcatgtc gtgtgcaatg cctatgaatt attacgtata cacagtgtgt cctcggcaat 360
 aacgaaagtc cgggaggggg cggggcggta ttcattgtgc agttgcccac aaattcaacg 420
 aaattgctac agtttttatt tgtaatgact gggcatggta agttaatatg attcttcata 480
 ctgattaagt gcttttgtta cttttttaat tattcaagta aaaatattaa tttgtgtttc 540
 atgggacttt ttgtagtagt taccctacta ctacattaaa cattaatttc aaagaagtag 600
 atatacgagt aaatgggcaa tatgaaaatt tgaaaaagg aaagcttatg atactaacta 660
 atgccaatg aaaactagga gtatgataat aatatgaaga tagccacca ggctatccca 720
 aaatcgatc caaatccaat ggtgttcatt aaattaggta atcgcatgtg ccttatgtc 780
 aaccatctc cgcctcaacc agtcatcttc ggtcgtgag gcaatcgaga tatggggcgc 840
 caccgacctt ggccaacatg ctccacattg ggctccaagt ggcaaccgca aaggtcacgc 900
 acagttcgcc attgcaatc gcatactgcc aatggaaact acattgcgta tctgggtggc 960
 ctttgatggc gctctaatta aaggctacct gccactaatt agtgatagac aatcgctggg 1020
 ggagttcggg tggcatcggt ggcaggcact taaccaaga cagggggggc aactggcatt 1080
 ggatggccgt ttttgaattc gtatgtcgga agcagtcgat gcagggttg gggggatgga 1140
 aacaaatgtt gtcaacgcca aaaccactga actgttaaaa gtgccattga atccaacaag 1200
 gatgctgggc gcaactgtgc aacctaaca actgtcgga agacagcagc aacatgggca 1260
 tgcattgctt gatactggga gtctgttcga tggatccac ttgaaccgaa cgtactgaa 1320
 ccgtgccccg gccagatgag gcgccccacc caacgccact cttgaaaacc ccaagccctt 1380
 tgcacgcgct aaatagtttt gttattgca cattgaaacc gagccagcga gcaattccgg 1440
 tggctgtcc gcgcgcgaca cactccagcg atctaactcag caatctcgac gacgaccggg 1500
 ctgacatggg gttttctcata cgctcgggta gacgcgacgt cgacgctcga tcgaatattt 1560
 tcccaatgca ctggcagaaa atgtgtggaa gtgtgagatt aagctcataa attagtagtg 1620
 cacttaaatg gaaaaatatt agaaacaaca gtgaacagtt gattgggtctt cttataaatt 1680
 ttattaatta ttgaacattt gaagaaagat attgattaaa tcaacttttg atgtatacat 1740
 atatataaaa aagtatatga tgactttcat gttgagaggt cataactttg caatgatatt 1800
 ggttctagtc atcatctcgt gaaacagctg tgcaagcatt cgattatatg tggtagtaa 1860

tttatttggg ttaatatatt ttctgcagtg tactgcttct gctgcgtcac ttcacattcg 1920
 tatcattttac atacgcagca ctgcggagtg agtcgctgag tacctggcgc tctggggtct 1980
 ctgggatctc tgggcttggg gatggatctc cactcgatga tctctccgcc tgggagccca 2040
 gatcatcgtc tgctatttgc aagtcgagag tcgcgcgagt cggacgtaca atcgccgcag 2100
 cggaatcaag tgtgataaaa gtgaacagaa ctttagccaa gtgcatttgg ctaatggaag 2160
 tgggtggcaaa agtcaaagcc acacgttata ctcgaaatcta aaaacaaata aataatgcat 2220
 aagcaggcga gtttgaagta attagcacia cgatgatgct ggcgggccaac tgaccacat 2280
 cgggaaatcg ctctaattca tatttgttgt cgagtgggcc aggataacag gataacagga 2340
 tactgctggc tcatattgcat ttgcatatat gcaaatagtt cgatctgcag gcgattgagt 2400
 gaccgaaagt gttggactgt gccaaatata taaccagcta acggggcaaaa agccactgaa 2460
 taaatggccc ttgttactcg gttcgtgtaa tgcgtctacg agtttagccc gtgttctgac 2520
 cgagaatcaa ttaaaattta ttgcacgagc atgccaaaca attcgcgggt gcagccacaa 2580
 aaacgcattc gaaaaacaat gccaccactc caatcacttg tgaccgcccc ccggctatgc 2640
 aaattagcca ttgcagcgat ttgtctaatt ctccagctaa acgctagtgg tgagttctca 2700
 gttggctaata atatatatat gtatatatat gaaatatgaa aaatcggaac acccctttgc 2760
 aaacattgct ccgcgcttag ctcatgatga tgccaattcc gagagcgttt tgaagatgca 2820
 ctgcgccattt gcattcaaaa gccaaagcga taaatggaga agcaaaaacca aaactgcata 2880
 gatcaattta caagtcggca aaggggttta ctgcgtgcac gtgcattgca gctgctatta 2940
 tagattttatt tattggcaaa caccctgaga acgagtttca ttggggggcc taagtgggag 3000
 aatgacctac acaggaaaagt gctcttaact aagcaactaa cttctggaaa agcgggaagt 3060
 gagagattaa gtactatctt atagatatgc cagaatatca aaaaagtatc taccagatac 3120
 cttgaaagat ctctgcatat ctcaattgca attcatgata agtttgttaa gttacgtttt 3180
 ttaatttcca attcaacctt tcaattagtt aataacgcca atctcagaca ttctaaacc 3240
 cctccctac ttaagggtaa atcccgatga tgcttgattg attttctcat tgetcagcta 3300
 tgcataaaaa tatcatatta attgatgagc acgagcttag ctaccagaat tgaaatccat 3360
 atgactgctc ggcaatttga aaaatgcgtt ggttcccagt catgcgcac cgttggatt 3420
 gaaaccaca ttcattggcat tccgttctgc ccccagttg cgctgctgct caagtgtccg 3480
 ttgcaccagt tgcagctgca gaagatcgtc ggattccggc caccgctgga gtatctgaat 3540
 gcggataatc ggatctacgg accggaaatg gtgagcaact tcaagactcg caacggccaa 3600
 caggaaattc cggtcagcca ggtgtgctgg cgcactgca acgaggatcc cgattgcatt 3660
 gcctatgtcc atctgctgga caggacgag tgccatggct actcgtactt cgagcgaacc 3720
 tcgcgctatc tgcccatctc ggtgaaatg cctctggtgg cagacggcga ggccgtcttc 3780
 tacgaaaaga cctgcctccg aggtgagtaa ttctccagcc aaacctccgg aagtggccgt 3840
 gatccgctc taatccattc cgacctgca gttcccagat cgtgccgtgg gcgtctctgg 3900
 gcactgacca aaatccccgg cagcacgctg gtctaccaca gcaagaagac catttcgacg 3960
 ctggtcacgc ggcgtgagt cgccgagcgc tgcttcttcg aaacctcagt ccgatgcctc 4020
 tccgcctcct ttgcgccctc ctatcggaac aatcgtgagc ggtaattgac tatttgttgt 4080
 ttgttgtttg ctatttgggt gtttgttgtt gtcggtgtgc agtgggtggg tgtttagatt 4140
 gctggtcgcc ggacaaatga atagcttttg ttgtgcattt ttaatgcatg gtcgagactt 4200
 ttcgccggat tatgacatca ctccgaggat ggtgatggga taggttagga ctattcaaca 4260
 atgtgtagca agctaataat atgataatat gatattataa tacgaaagaa agatatatcc 4320
 agaagacatc atcttttcga agctatgttc ttttccaaac aaatttttac aaaataagat 4380
 aagtattttt gaaaagttag atcatcagca atcatctaga ttttcttaa ctcaagtata 4440
 tatcgaattc ttctgaaata accgaactga cttggtcata atcgacacat catcgttttag 4500
 aagttaataa agcaaccttt aacctctcct tttcgtagct tccgcggcga ggccgggtcct 4560
 ggccagcgct cgtctccccg cctcggcaga tgtatgctga gcgacagggg caagaccgtc 4620
 cagccggacg cctttcgcgc ggctccatac gacgaggagt acatggagaa ccagtgccac 4680
 gaacgggcca tcgaaagtga caactgttcc tacgagctgt acgccaacag cagtttcatc 4740

tatgcgagg ccaggtat tt gggcctctcc caaaaagagg tgtgtccgcc gcgcttcgga 4800
 tgtcgcgc at tatgattgta atcgaaatgg atgggggggtc ggatgattga ttgatggctt 4860
 ctacctccgt attgcagtgt caggcgatgt gctcccacga ggcgaaagttc tactgccagg 4920
 gtgtctcctt ctactatgta aaccaactct cgctgtccga gtgtctcctc cactcggagg 4980
 acattgtatc cctgggtccg cgaagcctga agctccgtga aaactcgggtg taatgcgga 5040
 gggtaagtg cctggatggg aagatcttct ggggatgtgg tatgtcaat cttaatcgat 5100
 tccttattcc gcagtcggg ttttttgcac ccgcatgag atgaccatta agtacaatcc 5160
 caaggactgg ttctcggca agatctatgc cagcatgcac tccaaggact gcctggccag 5220
 aggatcgggc aatgggagtg ttctgtgac gctccagatc ggagcgagg taaaggagaa 5280
 ccgtgtggc atcctgcgtg cctacgaaat gacacaggaa taccaaaggt aagatgaagt 5340
 ccaatgtcca gtccattttt ttaattatat catttgcat atttagaacg tcatatctg 5400
 ctctggtggg catccaaaac aatccaaatg tgcaaaccca gggcgaccgg ctcatcaagg 5460
 ttggctgtat acagagcaat gccaccacat cgctggggtg ttgggttcgg gacagcagt 5520
 tggatagctc agagcctgtg ccagcgcca ttgactgga gtctcattg gactacacag 5580
 aacagtga gtattcttaa tagaatccct caaatgctt aattctatca caatcgatac 5640
 ctgcagcatg ttcccacag aggggtgtgt tctactaac agcagcactg ggccccatcc 5700
 gcatccagc atctcgcttc agattttgga tctatcccac cagcagcaga ccaacgacgt 5760
 gcagattgga cagaacctg aactacagat tgtggcgagg tacagcccac agcagttggc 5820
 agagcacatg gagttgcagc tggcaccact acccgacttt cgtgtacct cgctggtggc 5880
 caagacagcg gacaatgaga actttgtgt gctgatcgac gagcgaggat gtcccacaga 5940
 tgccagtgtg ttcccgtt tggaaaggg acacacagcc agcaggagca tgttgcgcgc 6000
 tcgcttccat gccttcaagt tctcaggaac ggccaacgta agcttcgatg taaagattcg 6060
 cttctgcgtg gagcgctgt cgcccagcaa ttgtattagt tcatcctggc aacggagaag 6120
 gcgacaggct gaccaaccag atcgtagacc ggaagacct cagagttcaga acccgtgtg 6180
 catctccacg gtggtggatg tggctccgca accagacaac ttaccagat cgaggagga 6240
 attgccccctc aactacaata tccgggtgca cggctccggac cagagcaaca ccaatagtta 6300
 tctgtacggc gagcggggag tgcgtctcat tgcgtgcata gacgaccgc tgcacctgga 6360
 taacgtttgc atcaaccaga gctgtgtgat tgcactgttc atcttctggc tgatctgtca 6420
 agttgccctg ctcttcggct gtggaatggg gctgcagcgc taccgcccgc tggccaagct 6480
 cgaggatgag cgacgcaggc tgcacgagga gtacctggag gcgaggagag tccactgggc 6540
 ggatcaaggc ggatacacac tctaattgac ggctggaacg caatgcgtat aaaatgcac 6600
 ttaatttaaat aaacataaat ctaacataaa tctaacaat gtttgcaacc gaggataagt 6660
 tcaggagttc ttcttgggat ggtagtgtc ccacttgcga tgggttagcg aattgaaatc 6720
 cgggcagtg tgagcattt tgcgcaaata gtcggacaac ttgagcagct cgggtgtcgt 6780
 gccacggttg agatgagcct gacggaatgg gcggatctt aggcgggact ttgggttcat 6840
 aaggaagttg cgacggatgt catcaaacat gatagtgtg ctcgagttgt attgcttga 6900
 cagggccagc attacaccaa gcggctttac gtccaccaca ccgcgtccg gcacatgaac 6960
 tgatatcatg gcggtggagt ccagatagaa catcacctg tagttatcgt tactggccac 7020
 gccagcagg cgcatctttt cctcgatcca gcgcatgctg gtggcgacc agatgacaat 7080
 gtcgtagtcc tcgtaggcgg aagtcagaaa ctctgtcaga tacggacgca ttagctccgt 7140
 gcctgtttca gcaggcgatc ggtgatcgaa tagggtatag tctatgtcca ggacaagcag 7200
 cttcttggcc tcacgcgcg gcgctaactc cttgatctg tagtctcgca cacgacgtg 7260
 caccttggcc aaatagacgg cggagtgtc cacggactct tcgcgttcat cggcgtcatc 7320
 gaagtcgtcg accacttcgc caatattatc gggcaggctg cacgcaccc ccatatcggc 7380
 ctctgtggag ccacacatca taagcttaaa gttgggctc agctccaaag cgctgatctt 7440
 cacattgtcg gctgtgtct ttctgcaag tcattggatc ttaaaactga aatatccga 7500
 agcctaggag tgcacgcac ctttgtact caggttgagc agcttttgac gttccggacg 7560
 cacctgtgtc ttgcggaata tctcgtgac cagcacttcc acggtgtcct ggtcggtgag 7620

gtccaccggg tactccttac cactccattt tacaatcact accacttctt tgacctccat 7680
 cttagctggt ttctattccg ctattaattt atcacaccat atatggtaat gtatgtttgt 7740
 tggatagaat ccagcaagtg gtttgcaata gtgtacctta aagatattaa ctaatttatt 7800
 agaagaccat ataaacagtc gagttgtcag aagtcgatag atactatcga ttgcaacgcc 7860
 cggcgttatc gattgcaatc ggcttgcaat aaaaataatg attttttgat tatatttttc 7920
 agagattatt aaaaaatatt tttaaattttt taaaattata tatttagcaa ttaaagaaag 7980
 tcatgcaaag acatgaggaa tgtccccaag ttgccaatag gcgattgttt cgccagttca 8040
 ttggccacac tggtcaccag ctgaaaacac aaaaaccgat cgtacagcat aaatttagct 8100
 cgaaaatgga ctaaacaag acagcgatcc ggaatccgag cggaacata gtctgcatga 8160
 actatctaac gatcctgctg tgcaaccgaa aaccgacgat gctctcgcg cggaacaagg 8220
 agaagtccca gcacaaggag ggcgtggtgg ggaagtacat gaagaaggac accccaccgg 8280
 atatttcggt gatcaatgtg tggagcgatc agcggggccaa gaagaaatcg ctgcagcgct 8340
 gtgcgagcac ctgcgccagc tgcgagttcc atccgcgcag ctgcagcacc agtcggaaca 8400
 cctactcctg caccgactcg cagccggact actaccatgc tcgacgagca cagagccaga 8460
 tgcccctgca gcagcactcc cactcgcate ctactctct gccccacccc tcccatccgc 8520
 atgtgcgtag tcactctccc ctgccgcccc accagttccg cgccagcagc aatcagttga 8580
 gtcagaacag cagcaactac gtaatttcg agcagatcga gcggatgcgc cgtcagcagt 8640
 cgtcgccact gctgcagacc acatcatcgc cggcgccggg agccggagga ttccagcgca 8700
 gctactccac caccagcgg cagcatcatc cccatctggg tgggtgacagc tacgatgcag 8760
 atcagggcct gctaagcgcc tcctatgcca acatgttgca actgccccag cggccacact 8820
 cgcccgtca ctacgccgtc ccgccgcagc agcagcagca tccacagatt catcaacagc 8880
 acgcctcgac gccgtttggc tccacgctgc ggttcgatcg agctgccatg tccatcaggg 8940
 agcgacagcc caggtatcag ccaactaggt aaactgcctc ttgaagtact atatttgaat 9000
 agatagcgg cgattgataa agtgggtaga gataatatga gcagctcttg attaaaggaa 9060
 taatccgtaa aaactacata ttgtcaaaaa gtgcttaata ttattataac ttttaaaaa 9120
 tgacaatgca cgaaatgttt tattttcgaa acatttattg ttcaaagatt ttttatttga 9180
 taacagattg ctttatttat ttacaataag aaaagttgat gtacaaaacc ggtttctact 9240
 cgctttacaa taattaaaac aataacacaa tatatgattt tctgtacgag gaatataatg 9300
 gaatatatat gatataata acatttttaa acacattttc tcttctgttt ccacagctct 9360
 ccgatgcagc agcaacaaca acaacaacaa cagcagcagc agcagctgca gcacacacaa 9420
 ctggcagctc acctgggcgg cagctactcc agcgattcgt acccgatcta cgagaatccg 9480
 tcccgcgtca tctcgatgcg cgccacgcag tcgcagcgat cggagtcgcc catctacagc 9540
 aatacgacgg cctcgtcggc cagctggcc gtggttccgc agcatcatca tcagggtcac 9600
 ctggcggtgc catctggaag cgggggagga tccctgagcg gcagcggtcg tgggtggcagt 9660
 tctggcagtg ttcgcggcgc ctctacctca gtgcaatcac tgtacgtccc accgcgaact 9720
 ccgccagtg cggttgccgg agcgggaggg agtgccaatg ggtcgtgca gaaggtaacca 9780
 tcacagcaat cgctcacgga gcccgaggag ctgcctctgc cgcccggtg ggccactcag 9840
 tacacgctac acggtcggaa atactatatt gatcacaaatg cgcataccac gcaactggaat 9900
 catccgttgg agcgcgaagg tctgccggtg ggctggcggc ggggtggtgc caagatgcat 9960
 ggcacctact atgagaacca gtataccggg cagagccaac gtcagcatcc atgcttgacc 10020
 tcctactatg tctacacgac gtctgcggag ccaccgaaag cgattcgacc agaggcgctg 10080
 ctctatgcc caccacgca cactcacaat gcactggtgc cggccaatcc ctatctgctc 10140
 gaggagatcc ccaagtgggt ggccgtctac tcggaggcgg actcgtccaa ggaccacctg 10200
 ctgcagttca acatgtttag cctgccggag ctggagggct tcgacagcat gctgggtcgg 10260
 ctcttcaagc aggaactggg caccatcgtg ggcctctacg agcgtaccg gtaagtgagc 10320
 ggccacatgc cgctgcattc tccgctctcc gaaaagccac tactctcttg ttacaccttt 10380
 cagtcgcgct ttgatactcg agaagaatcg acgcgcccggc cagaaccaga accaaaacca 10440
 gtgaccgggt gaccaggtga cgactgactc agaccacata ctgcgcagca gctatatgca 10500

catcatagtg ctccgtgaat cgacctttaa cttattttaac catcgactca tcgcgaaatc 10560
agtgcccttat acgaaaccag acgagatggg agccaagcag atccatgaca gttcgaatgc 10620
cttgatgaaa cgtagaattg tgctacgttc tatataacct taatgtgatt tgagcttggc 10680
gtttgtttgt aatgtgagca aagaaaatta aactgggtta ctgatcatct tacctgccga 10740
gcgcaattgt aatcgatgtg ccacctgaaa cccacacagg atttaacctg ggagtcgcat 10800
tcacgacggt atgttttggg aattcagcgc cgcgaagtgt aaataaaggg caacagttgg 10860
tgcccaagtc ttactcgact tggcttggca catatttccg agttccatgc caagttttcg 10920
attcgcttgc aaaaatttat cattgggcac aagtgaatcg tggccgattc tgtattggca 10980
aaaaaaaaa cagcgctcca atagaaagtg aatcttatgt ttgttttcgt ttggctatgc 11040
ttatttttag tcgaacctga taattcatte agtcgcctct tatcgatgc ttataaaact 11100
ttatagtcac tgtttctgca ggtccctcaa aaacagtttc tactgctgat aagaagtttt 11160
cgaagtctgg ggagtattcg gcattggaaa ggccaaaagt tgtgttttat tatattttga 11220
acatattaaa caggatacat aaaacgagag ttttagattg taattacatt tgcataatct 11280
tttgctaaat tgataagtaa acagaaaata tgactcgatg gatattattg actaataata 11340
tatatttagg ggtttggtat gattactttg tactgtgaga tacaagttcg tttgtccac 11400
agatactttt caattcatag cttatcctac agatacattt caattcatag cttatcccgt 11460
agatacattt ccattcattg cttatccac agatacattt tagcatattt tttttgaaat 11520
ttgaatttga aaaaaaagt tttttttttt ttttgttttg agaactactc gtcttgtaa 11580
aatattttaac tgttcccgcac tgaagtgtcc accttttcgg ccgccgggtt ctcaagtgc 11640
aaaataatgt ataataaaaa gccaaagata gtcggcggtc cgctctcgcc ccacttggtg 11700
ttgtgctgctc cgctgggtgc tcgctgccgc tgccgcagtc gacgtcgact ccactgctcc 11760
aatattttaa cggatccatt ggatcgcgca ctacgtcgca ctggagagtc gccatcgag 11820
ccatcatcat agcattccat tccacttgta gccatcggca gtcgctcaat cgtcagttgg 11880
gacacattat ttaacttcat tcttaacgtg agtgaattga tgtgttgggt ggcatcatg 11940
catatagcat aggcacaaca ctgttcta atccgattatc ttaatacaca taatccggcg 12000
gcttatacag atgttttgcg ttagcagttg gcggtctaaa gcctctgctt gccacatgc 12060
cagtgaagt tctaaccggt ctcaaacaga cgcacaaca gcgtatctcg tgcgtggaat 12120
catgaatgaa taaatgggtg ttactgttaa ctaacaatgg acctttttac caatcaatcg 12180
tcttatctat caccagaatt gaaacagaat tagtgaataa cttatgggtg atatcagttg 12240
aaacatgaag attcgtgtga acgatcgtga aagatatggg gttcgaactt taaattacc 12300
ttgtagttta ccactctcat tagttttgat ttatgtagaa ccaaaatttg gatcgtgact 12360
tgcgattagt attgcaatcg cagtgcattg cccaatctat tgattatctg caacttggtg 12420
cagactgccg caataattcg acggacacta tcagctagct ccattgattg agataagccc 12480
gttctcacgc ggtgttttac acttcttggc aatcgccaag tcacggccct cgcatataa 12540
aaaatatagt atgaacaatc gggaatcttt ttgttttacg atcgaccgac aaagcccatg 12600
tatttcctgt tacgtccatt tgggccatat aggcacataa aatgggtgct ccaacgcttg 12660
ccgtgggaaa gtgtgctcca attgcaaagt tgtaacattg agcgacattt gatgaagggt 12720
accgactttt atctcgacaa aaacacacac gaattccaga tgaagcgagc gtgcgtagtt 12780
tgcaactgaa gttttttttt tggacaatat agtttttatgt ttatatcatt ttatatcata 12840
ttatatctct tattgattga gtgtctgcac gggtcattaa attaagaagc aaaaaaaaaa 12900
aagggtgacg gaattgcatt ccatactcct acgagtagat atcaatttca cccgatcgtg 12960
gtcaattggg caattgaagt aattcacaat tgaatcaata caataccata tagggcttca 13020
ttgaagaaga tgccagcagg actggatgct catgcatgaa taagttgaac gttgaacgca 13080
agcagaatgg atttcagcac acaccgctg accactttgc tgcctcctct cctggccaca 13140
ggtgagatat cgcaatccag atattcgcat ctaataatga gggaaatttct cctgcccaca 13200
gttgccctgg gaaatgccc aagcagtcag ctcaccgtcg attcccatga catcaccgtt 13260
ctgctgaaca gcaacgagac ttttctggtg ttcgccaagt gagttgccat tgccgggaaa 13320
tccaaatcca aaacatatgg catcgtaaat ctattgtgcc cattacagcg gattgctaga 13380

cagcgacgtg gaagttgctg tgggaacaga ttcggaggat catttgctcc tcgatcccg 13440
 aacgtttgtg tatccagcgg gcagtactcg aaatcagtcg gtggtgataa ctggcctcaa 13500
 agccggcaac gtcaaagtgg tcgcagatag cgatgatgcg aacaaagaga tgtgagtaac 13560
 ttcacgggaa tcccaactgt tcccgtacct aattggaaaa ttcacttatt ttcagtggtg 13620
 aaggatgtgt tcgtacgcgt gactgtggcc aaatcgagag ctttgatcta cacctccatc 13680
 atctttggct gggtttactt tgtggcctgg tcggtgtcct tctatccgca gatctggagc 13740
 aactatcgcc gcaagtccgt cgagggactg aactttgatt tcctggccct caatatcggtg 13800
 ggcttcaccc tgtacagcat gttcaactgc ggctcttatt tcatcgagga tctgcagaac 13860
 gagtacgagg tgcgatatcc gctgggagtg aatcctgtga tgctcaacga cgtgggtcttc 13920
 tcaactgcat ccatgttcgc cacctgcatt acgatccttc agtgcttttt ctatcaggta 13980
 ataatatata tagcaaatac cattcaatag ccttatcgcc gaagtggcaa cagttgtcgc 14040
 attgaacact aattgccatc aatcaaatg ccaaatcatt tgaatcacag cggatagtta 14100
 cgatatgaag agtagataag gttttgactt gtaaaacatc catactttgt taaatttgtc 14160
 cagagagcac agcaaagggg gtcgttcatt gcctacggaa tattggccat cttcgccgtg 14220
 gtggtcgctg tgtctgccgg tttggccgga ggatccgtca tccattggct ggactttctg 14280
 tactactgca gttacgtcaa gctaaccatt accatcatca agtacgtgcc gcaagctctg 14340
 atgaactatc gccggaagag cacctccggc tggagcatcg gcaacattct gctggatttc 14400
 acgggaggaa cgctgagcat gctgcaaatg attctgaatg ctcataatta cggtaggata 14460
 tagtctatca atttgtgatt ttcgaatgaa atcgtgtctg gtttccagat gattgggtgt 14520
 cgattttcgg tgatcccacc aaattcggac tgggtctgtt ttccgtgctc ttcgatgtgt 14580
 tcttcatgct gcagcactat gtgttttaca ggtgattgaa acattgtgtg aatatgatac 14640
 ttaatctacg attatgtcat ctccactgta cacttatcat tattgtgtgt ctgttttcca 14700
 tttctcccca ggcattcgag ggaatcctcg agctctgacc tcaccaccgt gaccgatgtt 14760
 caaaatcgaa caaatgagtc gccgccgccc agcgaagtga cgactgagaa atattagagc 14820
 tgcattatca tatgtctgct gtagagaaaag acttttgtgc cagtagcgct ttatgtacat 14880
 ttttagaatt gtaaatatata ccgtatgccg tagctgccta agctttgtat aattcgtgctg 14940
 ttttaattga aatttagttt gactaaaatt tgggaatttca ccattaaata aaacttaatt 15000
 tttttagtag gccagaaatc atacgggtaca ttgctcgacc attcaaaggg ctgtgcagtg 15060
 aaaccaatth gctgcatacg gcgcgttatt tgcaaaactaa taaatagatt gaagtattga 15120
 aaaaatttca aaacagaaat tctaacttgc cgcacaatgg gcagcactgt tcgcactcgg 15180
 ccaaactcct atcgatagct tatcgatagc catggatata tgacattaag ttagccaatt 15240
 tccggttagt tgacatccct ggagcacgga agattcttgc ggacacaaat cgcaactgct 15300
 aaataaaatt tatttatttg agtgacacag catgagtcct cacaagtccg cgtcgttttag 15360
 cttgactttt aaccagtgag cggagatatt ttattcggct ttacccaaca aaataatggt 15420
 gcgccttttt gcagaaacac ttcgattgtt tcgcgtagca atagtcgcac aatttttgaa 15480
 gctttcaagg agttcctgga tttttgggat atcggaacg aagtttctgc agagtcagca 15540
 gttcgggtct ccagcaacgg agctttcaac ttgccgcaga gttttggcaa cgaatccaac 15600
 gaatatgccc acctggctac gcctgtggat ccagcctacg gaggaacaa cacgaacaac 15660
 atgatgcagt tcacgaacaa tctggaaatt ttggccaaca ataattccga tggcaataac 15720
 aaaattaatg catgcaacaa attcgtctgc cacaaggggt gagcaaattc aaaacacgcg 15780
 ctccaatcga taaacattgg ctacggcgat tgttcgcgct gcgtggcgaa tggcaaaatc 15840
 caaatagtcg gtggccacta cgattctgta gttttttgtt agcgaatttt taatathtag 15900
 cctccttccc caacaagatc gcttgatcag atatagccga ctaagatgta tatatcacag 15960
 ccaatgtcgt ggcacaaaga aaggtacagt gcggcaacaa attgatgatc gaacagtaga 16020
 aaccttgcat gtagcaacac gcttgtaact gcatcatcgc cgcggccaac ttgtttgtgt 16080
 ttgtttatcc agccaaggcg cagtttgcca ctaagttttt atttcccttt tacacttttag 16140
 cactgattcc gaggatgact ccacggaggt cgatatcaag gaggatattc cgaaaacggg 16200
 ggaggtatcg ggatcggaat tgtgagtacc tggtcacgtg gtcacatgtg gtttgccctg 16260

ttgctaacta ttattgtttt tattattcca ggaccacgga acccatggcc ttcttgacagg 16320
 gattaaacgt gagttgtgct tttaatgtgc aaagctatag cttactaact atttaatatc 16380
 attccccgca gtccgggaat ctgatgcagt tcagccagggt gggtaacatc gattagctat 16440
 tgcattctga agcgctggga cagatcgcc tgcacgagga tcagcaggaa gctggccacc 16500
 gccgagaaga cattgctgat cagtcgcatg tccagctcgt acaagcccaa gggtttaatt 16560
 tgggtacttg tcaccgtgac cagcagagta aagccgtgga ctgcctgacg gtagcggctg 16620
 tccgcatgct ggagattcat ctcttgaga atgactgccg atcttcgggt ggccaccaat 16680
 aggtgggtgc acaaatgcgt gagcaatgtg atctccgcca gcgagatgga gagggaaacc 16740
 agattgatca gcgatccaag accatcgtag ggcttgccca tgattaagggt gtccgctatg 16800
 gcatagtaca gactgtagaa acccaccgtt attccgagca ggtggcatat gagcgacaga 16860
 atcatggaca aggacattgg ggtagatata tttcccgaaat gcacatatat caacctatag 16920
 cgatacgcca gctggctgag ttcacccgcc aaggcgcaaa atcgctgcat gcggtagtag 16980
 ttagtgtaga acttttagctg gtcttctctc tgcagcagat tcacctctg cagctgcgct 17040
 tccagccgtc tggtcagagc gtacagaatc tccttcacca ccaccattgc gccaaagtag 17100
 cagttattga gaaaattcga aataattaag ggaaacagcc ggtacaagggt ccagatcaag 17160
 ctcatctcgg gatgctgccg cctctgttgc agtatgaaag ccacttcaat tgtagagga 17220
 aaagccacgg tcttgaccag agccaaaacg atggatatgt acagcgacct gctgtccaga 17280
 cggaattctt ttagggtagc aaagaagggc actttgtctc acaccttggc cacatgggtca 17340
 ctgattatca tttgcgacac atagttaata acagccaccg taatgttcat atagctgtac 17400
 agagtgggtg cgctcttcag gttgatctga ccctcctggt actccttgta gatttgccgc 17460
 ccgtaaacca agctgaatgc aattgccac agcgaagcaa aggcagatt tgcctttgag 17520
 aagcggaatc tttcacgacg gccgcccga tategattgg ccaggagtcc gaagacggtc 17580
 ataaagcta tcagtatgat cgtcagaaat ttcaccatac gccgatgcgc gtagtcgctg 17640
 gtgaagtcca tttctctcga acaattaata caaactgtga gcgcactttc cacagcatta 17700
 atatctgctt aattgttttc caactacca actgatgcca tctagaggac ctgtcaagta 17760
 gccggacact atcgggacac atcgcgaac gcatgtattt caccggccgt ccagaaacca 17820
 actgagcatg cggtgtgcta ctactagcca caaacaagag agcataagaa gcgtgaggga 17880
 agcggcattc cttgctgac tcagccgtg cctgcaattt cataagagcg acatgacgtc 17940
 aaagtcgctt cgaagttcac tttcagttgg aggacagaac aaaacactct tatctagccg 18000
 attagcacgg tgcactcctt cccgtcgtca tcgttttagcg agaatttcaa gcacttgtga 18060
 aaaatagaat agaatacaaa acaaatcgcc agtccatttg taactcgagc aagctggaac 18120
 atgaagctct atcagctcta tgagcgcaaa gtgtgaaccc ttatatgatt gcgagttaag 18180
 ttgacattca aataatatct tgtttttgct tacagcaatc cgtgctgcgc gaaatgatgc 18240
 tgcaggacat tcagatccag gcgaacacgc tgcccaagct agagaatcac aacatcggtg 18300
 gttattgctt cagcatgggt ctggatgagc cgcccaagtc tctttggatg tactcgattc 18360
 cgctgaacaa gctctacatc cggatgaaca aggccttcaa cgtggacgtt cagttcaagt 18420
 ctaaaatgcc catccaacca cttaatttgc gtgtgttctt ttgcttctcc aatgatgtga 18480
 gtgctcccgt ggtccgctgt caaaatcacc ttagcgttga gccttgtaag tgaagataac 18540
 aatacagatc gaacaggatt atttaactat catttgtaca aacctttagt gacggccaat 18600
 aacgcaaaaa tgcgcgagag cttgctgcgc agcgagaatc ccaacagtgt atattgtgga 18660
 aatgctcagg gcaagggaat ttccgagcgt ttttccgttg tagtccccct gaacatgagc 18720
 cggctctgtaa cccgcagtgg gctcacgcgc cagaccctgg ccttcaagtt cgtctgcaa 18780
 aactcgtgta tggggcgaaa agaaacttcc ttagtcttct gcctggagaa agcatggtaa 18840
 ggtgacagca aaactctaga tggctagaac aaagcttaac gtgttttctt tcttgacgcg 18900
 gcgatatcgt gggacagcat gttatcacatg ttaaaatatg tacgtgcccc aagcgggac 18960
 gcatccaaga cgaacgccag ctcaatagca agaagcgcaa gtccgtgccg gaagccgccg 19020
 aagaagatga gccgtccaag gtgcgtcggg gcattgctat aaagacggag gacacggaga 19080
 gcaatgatag ccgagactgc gacgactccg ccgagagtg gaacgtgtcg cggacaccgg 19140

atggcgatta cegtctggct attacgtgcc ccaataagga atggctgctg cagagcatcg 19200
 agggcatgat taaggaggcg gcggctgaag tcctgcgcaa tcccaaccaa gagaatctac 19260
 gtcgccatgc caacaaattg ctgagcctta agagtaagca gtgaatcgga ggacaaagag 19320
 attaagcttt acttaccgaa ctttcctttc agaacgtgcc tacgagctgc catgacttct 19380
 gatctggctg acaatctccc aggtatcaga tacctttgaa atgtgttgca tctgtggggt 19440
 atactacata gctattagta tcttaagttt gtattagtcc ttgttcgtaa ggcgtttaac 19500
 ggtgatattc cctttttggc atgttcgatg gccgaaaaga aaacattttt atatttttga 19560
 tagtatactg ttgttaactg cagttctatg tgactacgta acttttgtct accacaacaa 19620
 acatactctg taaaaaaaag ccaaaagtga atttattaaa gagttgtcat attttgcaaa 19680
 catatcctcg tgggtgtacg caatgccag agcctactgt acccccaccg tggagcacat 19740
 gctatgtgac atgtgtggct tgtgtgcggt caatgcactc aggatgcaac tcagctagct 19800
 agctgcta atgtcaaaat tgctgcgtcg cttttacata ctttatttat acccgatatct 19860
 gcacgtcttt ggttttagtt ctatgctttc aaaaaaaaaa aaacaacctc aagcagggcg 19920
 catgcgttgc gccagcgttg cacatgtgcg aggatgcaaa aaagtgcac aaacaccaga 19980
 tgttgacact gtgccgctgc agctgcaggc gacttttagct tttgccacat gcggcagcta 20040
 aatgtttact ctagcccacc gatcgtgtgt cattgacctt gggcaggggc attaatgtcg 20100
 ccctaactgt aacggaatga tagcctctgt gtccaaaaat tcagccaaag cggatgcact 20160
 cacttccatt tggggcctgt ccttcttcga ccggctgcca cttccactac cagtttgcca 20220
 ccacgaaaat gggctggtta aagtgtctca aaccagcgg agcaactcac tcaattctcg 20280
 ttggacgagc gcacagaaaa gtgggttttg atacgagttg agttcgagag acctttctgc 20340
 actgggaaca tacatgcggc tttgtgtaac agaataataa agtacgcaaa catatctgta 20400
 atacttaaag cacaaagaac aaatataaat gtatcataat ttgtttaatt atttattcga 20460
 ggtttccaaa caagtcattc tgataacaaa agttgtaaaa ataaaatcca ctaaaattaa 20520
 atatcaccca cttctcagaa taagcacagc tgtatatact tcagtatata tttttttcag 20580
 tgcacttttc ccaagcgtat caatcgcctt agaagcccaa ttaaatacgt ttctttgatt 20640
 ggcggttgcc aaaaggttga caattcgaaa gtggcgaca ctgggaggca gtgactcata 20700
 atttacataa ttatttcggg aagatattaa gactcatact atattcaagc agttgtttat 20760
 cattttaaac tggcagatac cccatcttta cggaccagat aaagggaag caaacacggc 20820
 tgggctctta tgggtacga tcttcacccg cagttccac tgtgcgctg gggaaaacaa 20880
 tatggcccaa acacataaaa aacaacaaaa aaaggaaaca accacagaaa gccgggctaa 20940
 gacgtcaggt gaaacgcagt agcttcactc gcgactcggc gcttccactc aaaggtgcta 21000
 ccgtgccca ctcaaatctg cagctcgtag atacgaaaac cagatagcgt cgagcggctg 21060
 gcgatcttca ctcaatgggg ggaaatactg ctatagagtc gaaagcttgt acacgtagtt 21120
 tggcattcgc agtcgcttgt tggcgttttt agtctgctgc ctgatcttcg acgcgtgca 21180
 gctgttttgg agtcgccg agtgccatat ttgctttgac cgcgaaaatt tctgggctaa 21240
 aaacagagat atttgagata cagatacata tatctcatac cacatattag ccaattgtgg 21300
 gtgcaacaag ctgtgagtga tgggtggagac ggcaacgaca acgaccataa cccgaccac 21360
 caccgcgctt ccggctggtg cagtaacggt aacaggaccc actgcctcgg ccacgcccac 21420
 cgcgacacag gcggccgcgc aggcgcactg caacgatgag accaccggg ccatcttcaa 21480
 tctgaaagtc atcgtctttc tgctcctcct gcctctggtc ctgctggccg tctttctcaa 21540
 gcacctgttg gattacctat tcgcgctggg actcaaggag aaggatgtca gtggcaagg 21600
 ggcactgggt agttgcattc gagtgcccat tggggctaac aaatggctgc aatgagcgtc 21660
 tggcaaatga gccattaata aggttagtca gatgcacatc agacatggat gcacttagaa 21720
 aatgcagtcg catttcatgt taagtactga cattaaaaaa gagatatatg tctgtgttta 21780
 gatacatctt tgggtaccaaa attaggttca gatacttcgt aaagaaattg gtaatggat 21840
 actttaatcg ttggcttcat gtgaatttgt tttcccagta tccgcttcta agtgatcttg 21900
 tatctgacga ctacttagcc aaccagaaac gtcacgcact ttccttttcc agtggctgcc 21960
 tccgggtttc caccacgcc accttgggt caccacctt tcccccttc ccgcttttct 22020

ttgcttttta tttctcctct tttttttttt tttgatgtca ctgccattag ggtgcggtcg 22080
 atcgcttagt actgtgttat taatgtaaat atttatgcgt ttggtgceca gcttggttag 22140
 ttgttggcca attgttttag tgtgtccaca gagccgcgtc tttggtgcca cggacagtta 22200
 atgtgacata atttcgctgt aagcgctgca atcaaagtga atctccagct gaaatcgtgc 22260
 tcatggcaac catatcgcgc tccaataatc acatatgcat cttggggcgt cgaattatgg 22320
 agaagtcaat tgccaatggg cgccaatgcc actggacaag gtcaagtgtat gatgccgtg 22380
 ccgatgctcc atatcgtaaa gaacctgatc gaattcggaa cccattagca tgcttttcag 22440
 gctttttata gtgggcgtgt gccggccata agcgtctcac gtagcgtatt aatgattcac 22500
 agcggcccca cttttgtttt agtctcagct ttttttttcg atcgttccct cagatatcgt 22560
 tttctcagat acagatacac atacagatac atttttgttg cggttgcaca gtggtatttt 22620
 cgggtggcag ggactggaga attcccatgc caactgttag cagcaactta attataagat 22680
 tgactttcgt tgataagttc tattgacatc atggttgctg aattcgagtt atttcagctc 22740
 aaaaataccc cttttttcga caccactggc caacggccaa ctgcaaactg gttttgctg 22800
 tgctgctata tttatttcca agatgaacga aaagagcgca aaaatgcaaa cctcagaaag 22860
 ttcacttttg ttttcagtct aatgtttgtg tttacaaaca atagagtgtga gaatttcgat 22920
 gggccaaagt atctgcaagt gtgtagcatg ccgggtatct ctcatagtcg tagataaaac 22980
 tcaactactg ttgcccgtgt taatttgcac atgatattga aattcttcgg ctgttctata 23040
 atcacaacaa ctgcgcattt gttattgttt tccccattgc tagtcgctaa cgtgccaaac 23100
 tctgaattga actcattccg gcttacattt cgattcaccc aactaccgca caccctaaac 23160
 gggcggtgag gtcacccagt gggcttcaat tacgggtcaaa agtcactcaa ttgtgcecca 23220
 gagggctcgc ccaccgagcg tatgagtaat gccattcata agtcgcctct gccgctgttg 23280
 ctgctgctca cataattgtc cgtaaatgag gtttttgttc aatgcgaagt cacattagct 23340
 cgagttgatt gtttgcaaat taagctaatt aatttacttg agtatacgag tgtaatgtga 23400
 gtaacctgtg atttaaaccc aggtgaccgg cggaggcagt gggctgggtc gcgagatctg 23460
 cttggaactg gcgcggcggg gctgcaagct ggccgctcgt gatgtcaact ccaagggatg 23520
 ttacgaaacg gtggagctgc tctccaagat tccacgctgc gttgccaagg cctacaaggt 23580
 gagttcacta gctgcttgga tatttaattg tttgataaca agaactttta ttccagaacg 23640
 acgtgtcatc gcctcgcgag ctccaactga tggccgceca ggtggagaag gaactgggtc 23700
 ccgtggacat tctggtcaac aatgcctccc tcatgcccac gacttcaaca cccagtctga 23760
 agagcgatga aatcgacaca atactgcagc tcaatctggg ctctacata atggtgagtg 23820
 tgtgcttctg aaaatgggac aaatataaaa cttcttgatt ttgcagacca ccaaggagtt 23880
 cctgccgaag atgataaaac gcaagtccgg tcatctggtg gcagtaaatg ccttagcggg 23940
 taagcttact tgggttaaagt gcttaccact tcattgatac ctatgtatat ataactcgca 24000
 tttaggctca gttccactgc caggagcggg catctacacg gccaccaa acggaatcga 24060
 gggcttcatg gaatcgctgc gagctgagct gcgattgtcc gactgtgact acgttcgcac 24120
 cacggtggcc aatgcctatc tgatgaggac cagcggagat cttccactgc tcagtgtatg 24180
 ggggtaagat tggtttatag tttgggcaga tcacttggtc tcatgcggt actacattta 24240
 gcattgccaa gagctatccc ggactgcccc caccatatgt ggccgagaag attgtcaagg 24300
 gcgtgttgct gaacgagcgc atggtgtatg tgccaaaaat attcgactc agtgtatggc 24360
 tgctcaggtg agaattgaat tagcccaggt aaccagcgat tatttctaac gattattgtt 24420
 gtcgccttgc tttagactgt tgcccaccaa gtggcaggat tacatgctgc ttcgcttcta 24480
 ccacttcgat gtgcgcagct cccacctgtt ttaactggaag tagggcacag gagaaggcac 24540
 atccccacc agaagcattt actcctgttt gtttcccaat tgcagttctt tattcaactg 24600
 ttgcttacgc taggtgtaca tgtttagcta tttatacgaa tctttaactt aaattaaatc 24660
 tatatcctaa cattagaatt acgtccggtt ggcctttcct attttatttc gtataagccg 24720
 aagttgttcg gagtagcaca tctctcggga ctgctggacg caggacctcc gttcgtagt 24780
 ccaagtgtag tcaagtggc atcgatggac cagcttgag cactggagc agtagtagaa 24840
 gtaggcgcag ttccgtggat gtggcataaa gccatagact ccctcctggc agttgatgat 24900

```

attctctcgc gtttgcacgc gattgcagga cactagatga gcaggagtac aggccttggc 24960
cagtccagcc cctcgttagc agaccatata aggataacat ggtccggcat tgggtaaaag 25020
tcgcagggta atcgccaatg gttccgcttt ctgagctggc ttcttgacca tcgaggggga 25080
tttagtggtt atgcctacgg gatcccggca tctcgacacc aacttttcgat ccaaacagcg 25140
ttccaatttt tcgtcgtagt aatgaccatc caagcactcg gcctcaaagg atcctggacc 25200
ggcacaatat atgtatttgg agcaattgct agagctggcg acataaactc ccaattgtgg 25260
agcactggca cactcttcga actccagggc actggatcga tgaccagca aggtcaccaa 25320
aataattgtt aagaaggtta cagctcccat ttcatttatt tttttaacga ccgaaatagc 25380
gggatgactt ctgtagactg acttcacga tgatgggttg agtatatttt tgcattgtct 25440
ccaactgata aagaagacaa gttattccat cgattactac gctgggttatc gtctggtaga 25500
taccgcta at gagcacatgg cagtaactgc cagccact ctggcggtc tcggtaattt 25560
gcattttcgt agcatacttc gcagcagcag caaagcaacc gattatttaa tgataccaca 25620
ccgcagcata atgctcgact gggcgccggt tcaataaaaa ttgaaaatgc actcaattcg 25680
caattaagtg tcgccacttc cgtacggaca agcggacaaa cggacggaca agcggacaaa 25740
tggacggata aacggacgga tggatggctg tcgaacgata ccattcaggc cattcaatcc 25800
attcatcgca gtcactctca ttattatttc catcgtcatc gtggctcgtt ctggctcgag 25860
ttaagcgatg gccatcgatt taatatccga tgagatattc ataacttgca attaggtttg 25920
gtggctctgc gctttacgta aatgattgct tagccgatta atgaagaatt accagtgcga 25980
atggctggga tctgtgggca ttatccaatt gaccaactac catgctaccc cactaccatt 26040
accattacca taatgtgcaa tgtgccaatt gggctcaaat taaaagtttt attaatgtc 26100
aattaaacgc tgctgcccag cagctgcttt gtggcataat ttttgggtca atctgcatat 26160
ctgattaaca ggttataacc ctcagtctac tacatatacc atgcaccaga tgcgcggggg 26220
cacagacaac aagaagtaaa agaaaggacc ccataatggtg ccgacggctc aagtgattaa 26280
gtgcacgacg agatcttcaa atgcagtgc acatgtgcac aaatacaaaa cacacacaca 26340
cacacacaca cagcatatt gaaaatgtat gtaaatctta attagattg tggatgaaga 26400
ccccagcac cttgatactt ctgctcaatg cgcattgcgc atgcgcagcc ccgcatccga 26460
agatccataa aaatagctca ctaattattt gtgtgctagg gttacagttc tcataaaaaa 26520
caaacaaact gtcgggcgtt ttatggatct tctgcctcta tggcctcaat gccccgcga 26580
agttttcgat ccccatcga ttcgaaaccg aagaagagct acgaccaatc acttttcaat 26640
tcctatgagc agttgagcat caattgattt cgatatgaaa ataaaatata tttatttatt 26700
atcacattac gtatcacagc cattcgcccg cctacgccct ggcattctgga tcgccacatc 26760
catcgtgcgg acctgtgcc ggcatttccg agctgattag cctccgaatc tcgaccagaa 26820
cccggctcgt tcgagcctcc aggttgctga gggcggtgtt taggtcatcc aagctggaat 26880
tgactctggc catcagacgc tccgagttgt tggtcagctc gatgaggtca tcgaaactgc 26940
tggcctggcg actctccatc gatatcctgt ccagatccag ctgcagctgc tcacggcg 27000
tgtccatctg ggctttaagg gctggaaaac aacttttcgat ttaaatttaa atttttttca 27060
ccctaaatca tgattttcgg tgttattttg tgccatgcga tccgaagtgt aaagcaaat 27120
tgacttgggt tgttttgcta tcgaacataa ttaaagttgc ttaccataaa ccaatttaat 27180
ttaattgtaa ttgcagctaa ctggcttttg ggtacttttg cttttaacgc caaatgtgaa 27240
atattaagta ttttttattt aagcgatggc acctgtaaat tgagatttaa gggggtatat 27300
taaattgggtg aacttgatga tttttttttt tcatcaaacg tttattaaag tctattgctt 27360
aaaaaatga aagtaaattg cttgccattt taggaggata tttttgaaaa atcgttacaa 27420
ctttt
27425

```

<210> 19

<211> 1781

<212> DNA

<213> *Drosophila melanogaster*

<400> 19

```

gaattcggca cgagacgcca tacaaaaagt tggaaactgag tggaaatcgga gtactatata 60
gccagccgat cccttccaga ggcgcggaag agtagctcac atccgaaccc acgtccccga 120
gccgatgtcg cggcgggaat agagcgattc gcagtccaaa cacgatgata aacccattg 180
catccgagtc ggaggccatc aattcggcca cctatgtgga caactatata gattcgggtg 240
aaaatctgcc ggacgacgtg cagcgccagt tgtcacgcat ccgcgacata gacgtccagt 300
acagaggcct cattcgcgac gtagaccact actacgacct gtatctgtcc ctgcagaact 360
ccgcggatgc cgggcgacgg tctcgaagca tctccaggat gcaccagagt ctcatcagg 420
cgcaggaact gggcgacgaa aaaatgcaga tcgtcaatca tatgcaggag ataatcgacg 480
gcaagctgcg ccagctggac accgaccagc agaacctgga cctgaaggag gaccgcgac 540
ggtatgcgct cctggacgat ggcacgcctt cgaagctgca acgcctgcag agcccgatga 600
gggagcaggg caaccaagcg ggcactggca acggtggcct aaatggaaac ggctgtctt 660
cggccaaaga tctgtacgcc ttgggcggct atgcagggtg tgtgtgcct gggttctaat 720
ccatgacctc cggcaacggt ggcggctcaa cgcaccaactc ggagcgctcg agccatgtca 780
gtaatggtgg caacagcggc tccaatggca atgccagcgg cggaggaggc ggagaactgc 840
agcgcacagg tagcaagcgg tcgaggaggc gaaacgagag tgtgtttaac aacggaagct 900
ctctggagat gggcggcaac gagtccaact cggcaaatga agccagtggc agtggtggtg 960
gcagtggcga gcgcaaatcc tcgttgggcg gtgccagtgg agcgggacag ggacgaaagg 1020
ccagtctgca gtccgcttct ggcagtgttg ctagcggctc tgcagccacg agcagtggag 1080
cagccggagg tgggtggtgcc aacggagccg gcgtagtgtg tggcaataat tccggcaaga 1140
agaaaaagcg caaggtacgc ggttctgggg cttcaaagtc caatgccagt acgcgagagg 1200
agacgccgcc gccggagacc attgatccgg acgagccgac ctactgtgtc tgcaatcaga 1260
tctcctttgg cgagatgata ctgtgcgaca atgacctgtg ccccatcgag tggttccatt 1320
ttctgtgctg ctccctggta ctaaaaccaa aaggcaagtg gttctgcccc aactgccgcg 1380
gagaacggcc aaatgtaatg aaacccaagg cgcagttcct caaagaactg gagcgctaca 1440
acaaggaaaa ggaggagaag acctagtcta ttaggcagc ctatccaacc cattgctctg 1500
tgtctaacac caggctctgt aaaatattcg atcctaagat ttaccttaat gtatatttag 1560
tgactttctt agaccgatac ccttttcgac tttccctctt ttcacccagt ttagatccct 1620
cgcttctatg gttataggtc gtcagttttc atttaaagtt tctgtacaaa caatatcttt 1680
ctcaatgtaa acacacaaaa actcgtataa ttagagtaca cctaaactta atttatggta 1740
ataaacgttg atattcaaaa aaaaaaaaaa aaaaaactcg a 1781

```

<210> 20

<211> 433

<212> PRT

<213> *Drosophila melanogaster*

<400> 20

```

Met Ile Asn Pro Ile Ala Ser Glu Ser Glu Ala Ile Asn Ser Ala Thr
  1             5             10             15

```

```

Tyr Val Asp Asn Tyr Ile Asp Ser Val Glu Asn Leu Pro Asp Asp Val
      20             25             30

```

```

Gln Arg Gln Leu Ser Arg Ile Arg Asp Ile Asp Val Gln Tyr Arg Gly

```

35 40 45
 Leu Ile Arg Asp Val Asp His Tyr Tyr Asp Leu Tyr Leu Ser Leu Gln
 50 55 60
 Asn Ser Ala Asp Ala Gly Arg Arg Ser Arg Ser Ile Ser Arg Met His
 65 70 75 80
 Gln Ser Leu Ile Gln Ala Gln Glu Leu Gly Asp Glu Lys Met Gln Ile
 85 90 95
 Val Asn His Met Gln Glu Ile Ile Asp Gly Lys Leu Arg Gln Leu Asp
 100 105 110
 Thr Asp Gln Gln Asn Leu Asp Leu Lys Glu Asp Arg Asp Arg Tyr Ala
 115 120 125
 Leu Leu Asp Asp Gly Thr Pro Ser Lys Leu Gln Arg Leu Gln Ser Pro
 130 135 140
 Met Arg Glu Gln Gly Asn Gln Ala Gly Thr Gly Asn Gly Gly Leu Asn
 145 150 155 160
 Gly Asn Gly Leu Leu Ser Ala Lys Asp Leu Tyr Ala Leu Gly Gly Tyr
 165 170 175
 Ala Gly Gly Val Val Pro Gly Ser Asn Ala Met Thr Ser Gly Asn Gly
 180 185 190
 Gly Gly Ser Thr Pro Asn Ser Glu Arg Ser Ser His Val Ser Asn Gly
 195 200 205
 Gly Asn Ser Gly Ser Asn Gly Asn Ala Ser Gly Gly Gly Gly Gly Glu
 210 215 220
 Leu Gln Arg Thr Gly Ser Lys Arg Ser Arg Arg Arg Asn Glu Ser Val
 225 230 235 240
 Val Asn Asn Gly Ser Ser Leu Glu Met Gly Gly Asn Glu Ser Asn Ser
 245 250 255
 Ala Asn Glu Ala Ser Gly Ser Gly Gly Gly Ser Gly Glu Arg Lys Ser
 260 265 270
 Ser Leu Gly Gly Ala Ser Gly Ala Gly Gln Gly Arg Lys Ala Ser Leu
 275 280 285
 Gln Ser Ala Ser Gly Ser Leu Ala Ser Gly Ser Ala Ala Thr Ser Ser

290 295 300
 Gly Ala Ala Gly Gly Gly Gly Ala Asn Gly Ala Gly Val Val Gly Gly
 305 310 315 320
 Asn Asn Ser Gly Lys Lys Lys Lys Arg Lys Val Arg Gly Ser Gly Ala
 325 330 335
 Ser Asn Ala Asn Ala Ser Thr Arg Glu Glu Thr Pro Pro Pro Glu Thr
 340 345 350
 Ile Asp Pro Asp Glu Pro Thr Tyr Cys Val Cys Asn Gln Ile Ser Phe
 355 360 365
 Gly Glu Met Ile Leu Cys Asp Asn Asp Leu Cys Pro Ile Glu Trp Phe
 370 375 380
 His Phe Ser Cys Val Ser Leu Val Leu Lys Pro Lys Gly Lys Trp Phe
 385 390 395 400
 Cys Pro Asn Cys Arg Gly Glu Arg Pro Asn Val Met Lys Pro Lys Ala
 405 410 415
 Gln Phe Leu Lys Glu Leu Glu Arg Tyr Asn Lys Glu Lys Glu Glu Lys
 420 425 430

Thr

<210> 21

<211> 2666

<212> DNA

<213> *Drosophila melanogaster*

<400> 21

cttttgtac agtctaaacg gggattcgcg taaactacgc agaaatataa acaaacaaaa 60
 actagtagac tatagaatat aaacagtttc ctaccaatgg agacttgtga agtggaggga 120
 gaggcggaga cgctggtgag acgcttctcc gtcagctgcg agcaattgga gctggaagcg 180
 agaattcagc aaagcgctct gtccacctac catcgcttgg atgcggtcaa cgggctgtcc 240
 accagcgagg cagatgccca ggagtggctg tgttgccgct tctacagcga actgcagcgc 300
 tcgaagatgc gcgatattag ggagtccatc aacgaggcaa acgattcggg ggccaagaac 360
 tgctgctgga acgtgtcact aaccgcgtctg ctgcgcagct ttaagatgaa cgtgtcccag 420
 tttctacgcc gcatggagca ctggaattgg ctgacccaaa acgagaacac tttccagctg 480
 gaggttgagg aactgcgttg tcgacttggg attacttga cgctgctgcg gcattataag 540
 cacatctttc ggagcctgtt cgttcacccg gcaagggtgc ggaccgggt gccgcgaatc 600
 actaccaagc gctgtatgag ttcggttggg tgccttcct ggctattcgc aacgagttac 660
 ccgggtttgc gattacaaac ctgatcaacg gctgtcaggt gctcgtttgc acaatggatc 720


```

tccttttcgt gaacgcctta gaggtgcccc gatccgtagt tatccgccgg gagttctctg 780
gagtgcceaa gaattgggac accgaagact tcaatcctat tttgctaaat aaatatagcg 840
tgctagaagc actgggagaa ctgattcccc agctaccagc gaagggagtg gtgcaaatga 900
agaacgcctt tttccacaaa gccttaataa tgctctatat ggaccatagt ctagttagag 960
acgacaccca tatgcgggag atcattaagg agggtagtct agatatcaat ctggaaaact 1020
taaatcgcaa atacaccaat caagtagccg acattagtga gatggacgag cgtgtgctgc 1080
tcagcgtcca gggggcgata gagaccaaag gggactctcc taaaagccca cagctcgctt 1140
tccaaacaag ctgctcacct tcgcatagga agctgtccac ccatgatcta ccagcaagtc 1200
ttcccctaag cattataaaa gcattcccca agaaggaaga cgcagataaa attgtaaatt 1260
atntagatca aactctggaa gaaatgaatc ggacctttac catggccgtg aaagattttt 1320
tggatgctaa gttgtctgga aaacgattcc gccaggccag aggcctttac tacaatat 1380
tgcagaaaat tttgggaccg gagctggttc aaaaaccaca gctgaagatt ggtcagttaa 1440
tgaagcagcg caagcttacc gccgcctgt tagcttgctg cctggaactg gcacttcacg 1500
tccaccacaa actagtggaa ggcctaaggt ttccctttgt cctgcactgc ttttcaactg 1560
acgcctacga ctttcaaaaag attctagagt tgggtggtgc ctacgatcat ggttttctg 1620
gcagagagct gatcaagcac ctggatgtgg tggaggaaat gtgcctggag tcgttgattt 1680
tccgcaagag ctacacagct tgggtgggagc taaatcaaag acttccccgc tacaaggaag 1740
tcgatgcaga aacagaagac aaggagaact tttcaacagg ctcaagcatc tgccttcgaa 1800
agttctacgg actggccaac cggcggtgc tccttctgtg taagagtctt tgcctcgtg 1860
attcctttcc ccaaatatgg cacctggccg agcactcttt caccttagag agtagccgtc 1920
tgctccgcaa tcgacacctg gaccaactgc tgttggtgcgc catacatctt catgttcggc 1980
tcgagaagct tcacctact ttacagcatga ttatccagca ctatcgccga cagccgact 2040
ttcggagaag cgcttaccga gaggttagct tgggcaatgg tcagaccgct gatattatca 2100
ctttctacaa cagtgtgtat gtccaaagta tgggcaacta tggccgccac ctggagtgtg 2160
cgcaaacacg caagtcactg gaagaatcac agagtagcgt tggattctg acggaaaaca 2220
acttccaacg aattgagcat gagagccaac atcagcatat cttcaccgcc cctcccagg 2280
gtatgccaaa gtggctcctg ctccagtcac ccaccttcac cteccgccgc atcaccactt 2340
tccttgcaaa gctcgcccaa cgtaaagcgt gctgcttcga gtaacgactt gatgagagag 2400
atcaagcgac caaacatcct gcggcgctgc cagctttcag tgatctaata accaatcaaa 2460
aaaggcttaa atacttggct gcattttacg cagctagctt agtatatttc ttaaactcaa 2520
aaatggtaat taaataatgt ttaaattata gatattttat taacttgctc aagtaagtta 2580
aaagcttttg cttttgtaaa aataaaggaa taactgccac tcgtagttaa aataaatttt 2640
taaaaaaaaa aaaaaaaaaa ctcgag 2666

```

<210> 22

<211> 556

<212> PRT

<213> *Drosophila melanogaster*

<400> 22

```

Met Asp Leu Leu Phe Val Asn Ala Leu Glu Val Pro Arg Ser Val Val
  1              5              10             15

```

```

Ile Arg Arg Glu Phe Ser Gly Val Pro Lys Asn Trp Asp Thr Glu Asp
      20              25             30

```

```

Phe Asn Pro Ile Leu Leu Asn Lys Tyr Ser Val Leu Glu Ala Leu Gly

```

35 40 45
 Glu Leu Ile Pro Glu Leu Pro Ala Lys Gly Val Val Gln Met Lys Asn
 50 55 60
 Ala Phe Phe His Lys Ala Leu Ile Met Leu Tyr Met Asp His Ser Leu
 65 70 75 80
 Val Gly Asp Asp Thr His Met Arg Glu Ile Ile Lys Glu Gly Met Leu
 85 90 95
 Asp Ile Asn Leu Glu Asn Leu Asn Arg Lys Tyr Thr Asn Gln Val Ala
 100 105 110
 Asp Ile Ser Glu Met Asp Glu Arg Val Leu Leu Ser Val Gln Gly Ala
 115 120 125
 Ile Glu Thr Lys Gly Asp Ser Pro Lys Ser Pro Gln Leu Ala Phe Gln
 130 135 140
 Thr Ser Ser Ser Pro Ser His Arg Lys Leu Ser Thr His Asp Leu Pro
 145 150 155 160
 Ala Ser Leu Pro Leu Ser Ile Ile Lys Ala Phe Pro Lys Lys Glu Asp
 165 170 175
 Ala Asp Lys Ile Val Asn Tyr Leu Asp Gln Thr Leu Glu Glu Met Asn
 180 185 190
 Arg Thr Phe Thr Met Ala Val Lys Asp Phe Leu Asp Ala Lys Leu Ser
 195 200 205
 Gly Lys Arg Phe Arg Gln Ala Arg Gly Leu Tyr Tyr Lys Tyr Leu Gln
 210 215 220
 Lys Ile Leu Gly Pro Glu Leu Val Gln Lys Pro Gln Leu Lys Ile Gly
 225 230 235 240
 Gln Leu Met Lys Gln Arg Lys Leu Thr Ala Ala Leu Leu Ala Cys Cys
 245 250 255
 Leu Glu Leu Ala Leu His Val His His Lys Leu Val Glu Gly Leu Arg
 260 265 270
 Phe Pro Phe Val Leu His Cys Phe Ser Leu Asp Ala Tyr Asp Phe Gln
 275 280 285
 Lys Ile Leu Glu Leu Val Val Arg Tyr Asp His Gly Phe Leu Gly Arg

290 295 300
 Glu Leu Ile Lys His Leu Asp Val Val Glu Glu Met Cys Leu Glu Ser
 305 310 315 320
 Leu Ile Phe Arg Lys Ser Ser Gln Leu Trp Trp Glu Leu Asn Gln Arg
 325 330 335
 Leu Pro Arg Tyr Lys Glu Val Asp Ala Glu Thr Glu Asp Lys Glu Asn
 340 345 350
 Phe Ser Thr Gly Ser Ser Ile Cys Leu Arg Lys Phe Tyr Gly Leu Ala
 355 360 365
 Asn Arg Arg Leu Leu Leu Leu Cys Lys Ser Leu Cys Leu Val Asp Ser
 370 375 380
 Phe Pro Gln Ile Trp His Leu Ala Glu His Ser Phe Thr Leu Glu Ser
 385 390 395 400
 Ser Arg Leu Leu Arg Asn Arg His Leu Asp Gln Leu Leu Leu Cys Ala
 405 410 415
 Ile His Leu His Val Arg Leu Glu Lys Leu His Leu Thr Phe Ser Met
 420 425 430
 Ile Ile Gln His Tyr Arg Arg Gln Pro His Phe Arg Arg Ser Ala Tyr
 435 440 445
 Arg Glu Val Ser Leu Gly Asn Gly Gln Thr Ala Asp Ile Ile Thr Phe
 450 455 460
 Tyr Asn Ser Val Tyr Val Gln Ser Met Gly Asn Tyr Gly Arg His Leu
 465 470 475 480
 Glu Cys Ala Gln Thr Arg Lys Ser Leu Glu Glu Ser Gln Ser Ser Val
 485 490 495
 Gly Ile Leu Thr Glu Asn Asn Phe Gln Arg Ile Glu His Glu Ser Gln
 500 505 510
 His Gln His Ile Phe Thr Ala Pro Ser Gln Gly Met Pro Lys Trp Leu
 515 520 525
 Leu Leu Gln Ser Ser Thr Phe Ile Ser Arg Arg Ile Thr Thr Phe Leu
 530 535 540
 Ala Lys Leu Ala Gln Arg Lys Ala Cys Cys Phe Glu

545

550

555

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/06602

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C07K 14/00; C12N 15/00

US CL : 435/455; 530/350; 536/23.5; 800/3, 13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455; 530/350; 536/23.5; 800/3, 13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST

Eialog (file: medicine)


search terms: p53, Rb, tumor suppressor, Drosophila, insect.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DONEHOWER et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 19 March 1992, Vol. 356, pages 215-221, entire document.	1-28
A	FIELDS et al. Presence of a potent transcription activating sequence in the p53 protein. Science. 31 August 1990, Vol. 249, pages 1046-1049, entire document.	1-28
A	KUSSIE et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science. 08 November 1996, Vol. 274, pages 948-953, entire document.	1-28

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
Q document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 19 JUNE 2000	Date of mailing of the international search report 09 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE-MARIE BAKER, PH.D.  Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06602

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEVINE, A. J. p53, the cellular gatekeeper for growth and division. Cell. 07 February 1997, Vol. 88, pages 323-331, entire document.	1-28
A	RAYCROFT et al. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science. 31 August 1990, Vol. 249, pages 1049-1051, entire document.	1-28

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 September 2000 (21.09.2000)

PCT

(10) International Publication Number
WO 00/55178 A1

(51) International Patent Classification⁷: C07H 21/04,
C07K 14/00, C12N 15/00

Burlingame, CA 94010 (US). ROBERTSON, Stephanie,
A.; 255 Fowler Avenue, San Francisco, CA 94127 (US).

(21) International Application Number: PCT/US00/06602

(74) Agent: BRUNELLE, Jan, P.; Exelixis, Inc., 280 East
Grand Avenue, South San Francisco, CA 94080 (US).

(22) International Filing Date: 13 March 2000 (13.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/268,969 16 March 1999 (16.03.1999) US
60/184,373 23 February 2000 (23.02.2000) US

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,
UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant: EXELIXIS, INC. [US/US]; 280 East Grand
Avenue, South San Francisco, CA 94080 (US).

(72) Inventors: BUCHMAN, Andrew, Roy; 3119 Epton
Avenue, Berkeley, CA 94705 (US). PLATT, Darren,
Mark; 929 Pine Street, Apt. 201, San Francisco, CA
94108 (US). OLLMAN, Michael, Martin; 1805 Atschul
Avenue, Menlo Park, CA 94025 (US). YOUNG, Lynn,
Marie; 250 Baldwin Avenue, #4, San Mateo, CA 94401
(US). DEMSKY, Madelyn, Robin; 1770 Pine Street,
3203, San Francisco, CA 94109 (US). KEEGAN, Kevin,
Patrick; 17311 Via Estrella, San Lorenzo, CA 94580
(US). FRIEDMAN, Lori; One Bayside Village Place,
Unit 212, San Francisco, CA 94107 (US). KOPCZYNSKI,
Casey; 2769 St. James Road, Belmont, CA 94002
(US). LARSON, Jeffrey, S.; 1220 El Camino Real #305,

Published:
— with international search report

(48) Date of publication of this corrected version:
25 April 2002

(15) Information about Correction:
see PCT Gazette No. 17/2002 of 25 April 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: INSECT p53 TUMOR SUPPRESSOR GENES AND PROTEINS

(57) Abstract: A family of p53 tumor suppressor nucleic acid and protein isolated from several insect species is described. The p53 nucleic acid and protein can be used to genetically modify metazoan invertebrate organisms, such as insects and worms, or cultured cells, resulting in p53 expression or mis-expression. The genetically modified organisms or cells can be used in screening assays to identify candidate compounds that are potential pesticidal agents or therapeutics that interact with p53 protein. They can also be used in methods for studying p53 activity and identifying other genes that modulate the function of, or interact with, the p53 gene. Nucleic acid and protein sequences for *Drosophila* p33 and Rb tumor suppressors are also described.

WO 00/55178 A1

INSECT p53 TUMOR SUPPRESSOR GENES AND PROTEINS

REFERENCE TO RELATED APPLICATION

5 This application is a continuation-in-part of U.S. application no. 09/268,969, filed March 16, 1999; and of U.S. application no. 60/184,373 of same title, filed February 23, 2000. The entire contents of both prior applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10 The p53 gene is mutated in over 50 different types of human cancers, including familial and spontaneous cancers, and is believed to be the most commonly mutated gene in human cancer (Zambetti and Levine, FASEB (1993) 7:855-865; Hollstein, *et al.*, Nucleic Acids Res. (1994) 22:3551-3555). Greater than 90% of mutations in the p53 gene are missense mutations that alter a single amino acid that inactivates p53 function. Aberrant
15 forms of human p53 are associated with poor prognosis, more aggressive tumors, metastasis, and survival rates of less than 5 years (Koshland, Science (1993) 262:1953).

 The human p53 protein normally functions as a central integrator of signals arising from different forms of cellular stress, including DNA damage, hypoxia, nucleotide deprivation, and oncogene activation (Prives, Cell (1998) 95:5-8). In response to these
20 signals, p53 protein levels are greatly increased with the result that the accumulated p53 activates pathways of cell cycle arrest or apoptosis depending on the nature and strength of these signals. Indeed, multiple lines of experimental evidence have pointed to a key role for p53 as a tumor suppressor (Levine, Cell (1997) 88:323-331). For example, homozygous p53 "knockout" mice are developmentally normal but exhibit nearly 100% incidence of
25 neoplasia in the first year of life (Donehower *et al.*, Nature (1992) 356:215-221). The biochemical mechanisms and pathways through which p53 functions in normal and cancerous cells are not fully understood, but one clearly important aspect of p53 function is its activity as a gene-specific transcriptional activator. Among the genes with known p53-response elements are several with well-characterized roles in either regulation of the cell
30 cycle or apoptosis, including GADD45, p21/Waf1/Cip1, cyclin G, Bax, IGF-BP3, and MDM2 (Levine, Cell (1997) 88:323-331).

Human p53 is a 393 amino acid phosphoprotein which is divided structurally and functionally into distinct domains joined in the following order from N-terminus to C-terminus of the polypeptide chain: (a) a transcriptional activation domain; (b) a sequence-specific DNA-binding domain; (c) a linker domain; (d) an oligomerization domain; and (e) a basic regulatory domain. Other structural details of the p53 protein are in keeping with its function as a sequence-specific gene activator that responds to a variety of stress signals. For example, the most N-terminal domain of p53 is rich in acidic residues, consistent with structural features of other transcriptional activators (Fields and Jang, Science (1990) 249:1046-49). By contrast, the most C-terminal domain of p53 is rich in basic residues, and has the ability to bind single-stranded DNA, double-stranded DNA ends, and internal deletions loops (Jayaraman and Prives, Cell (1995) 81: 1021-1029). The association of the p53 C-terminal basic regulatory domain with these forms of DNA that are generated during DNA repair may trigger conversion of p53 from a latent to an activated state capable of site-specific DNA binding to target genes (Hupp and Lane, Curr. Biol. (1994) 4: 865-875), thereby providing one mechanism to regulate p53 function in response to DNA damage. Importantly, both the N-terminal activation domain and the C-terminal basic regulatory domain of p53 are subject to numerous covalent modifications which correlate with stress-induced signals (Prives, Cell (1998) 95:5-8). For example, the N-terminal activation domain contains residues that are targets for phosphorylation by the DNA-activated protein kinase, the ATM kinase, and the cyclin activated kinase complex. The C-terminal basic regulatory domain contains residues that are targets for phosphorylation by protein kinase-C, cyclin dependent kinase, and casein kinase II, as well as residues that are targets for acetylation by PCAF and p300 acetyl transferases. p53 activity is also modulated by specific non-covalent protein-protein interactions (Ko and Prives, Genes Dev. (1996) 10: 1054-1072). Most notably, the MDM2 protein binds a short, highly conserved protein sequence motif, residues 13-29, in the N-terminal activation domain of p53 (Kussie *et al.*, Science (1996) 274:948-953. As a result of binding p53, MDM2 both represses p53 transcriptional activity and promotes the degradation of p53.

Although several mammalian and vertebrate homologs of the tumor suppressor p53 have been described, only two invertebrate homologs have been identified to date in mollusc and squid. Few lines of evidence, however, have hinted at the existence of a p53 homolog in any other invertebrate species, such as the fruit fly *Drosophila*. Indeed, numerous direct attempts to isolate a *Drosophila* p53 gene by either cross-hybridization or PCR have failed to identify a p53-like gene in this species (Soussi *et al.*, Oncogene (1990)

5: 945-952). However, other studies of response to DNA damage in insect cells using nucleic cross-hybridization and antibody cross-reactivity have provided suggestive evidence for existence of p53-, p21-, and MDM2-like genes (Bae *et al.*, Exp Cell Res (1995) 375:105-106; Yakes, 1994, Ph.D. thesis, Wayne State University). Nonetheless, no isolated insect p53 genes or proteins have been reported to date.

Identification of novel p53 orthologues in model organisms such as *Drosophila melanogaster* and other insect species provides important and useful tools for genetic and molecular study and validation of these molecules as potential pharmaceutical and pesticide targets. The present invention discloses insect p53 genes and proteins from a variety of diverse insect species. In addition, *Drosophila* homologs of p33 and Rb genes, which are also involved in tumor suppression, are described.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide insect p53 nucleic acid and protein sequences that can be used in genetic screening methods to characterize pathways that p53 may be involved in as well as other interacting genetic pathways. It is also an object of the invention to provide methods for screening compounds that interact with p53 such as those that may have utility as therapeutics.

These and other objects are provided by the present invention which concerns the identification and characterization of insect p53 genes and proteins in a variety of insect species. Isolated nucleic acid molecules are provided that comprise nucleic acid sequences encoding p53 polypeptides and derivatives thereof. Vectors and host cells comprising the p53 nucleic acid molecules are also described, as well as metazoan invertebrate organisms (*e.g.* insects, coelomates and pseudocoelomates) that are genetically modified to express or mis-express a p53 protein.

An important utility of the insect p53 nucleic acids and proteins is that they can be used in screening assays to identify candidate compounds which are potential therapeutics or pesticides that interact with p53 proteins. Such assays typically comprise contacting a p53 polypeptide with one or more candidate molecules, and detecting any interaction between the candidate compound and the p53 polypeptide. The assays may comprise adding the candidate molecules to cultures of cells genetically engineered to express p53 proteins, or alternatively, administering the candidate compound to a metazoan invertebrate organism genetically engineered to express p53 protein.

The genetically engineered metazoan invertebrate animals of the invention can also be used in methods for studying p53 activity, or for validating therapeutic or pesticidal strategies based on manipulation of the p53 pathway. These methods typically involve detecting the phenotype caused by the expression or mis-expression of the p53 protein. The methods may additionally comprise observing a second animal that has the same genetic modification as the first animal and, additionally has a mutation in a gene of interest. Any difference between the phenotypes of the two animals identifies the gene of interest as capable of modifying the function of the gene encoding the p53 protein.

10

BRIEF DESCRIPTION OF THE FIGURE

Figures 1A-1B show a CLUSTALW alignment of the amino acid sequences of the insect p53 proteins identified from *Drosophila*, *Leptinotarsa*, *Tribolium*, and *Heliothis*, with p53 sequences previously identified in human, *Xenopus*, and squid. Identical amino acid residues within the alignment are grouped within solid lines and similar amino acid residues are grouped within dashed lines.

15

DETAILED DESCRIPTION OF THE INVENTION

The use of invertebrate model organism genetics and related technologies can greatly facilitate the elucidation of biological pathways (Scangos, Nat. Biotechnol. (1997) 15:1220-1221; Margolis and Duyk, Nature Biotech. (1998) 16:311). Of particular use is the insect model organism, *Drosophila melanogaster* (hereinafter referred to generally as "*Drosophila*"). An extensive search for p53 nucleic acid and its encoded protein in *Drosophila* was conducted in an attempt to identify new and useful tools for probing the function and regulation of the p53 genes, and for use as targets in drug discovery. p53 nucleic acid has also been identified in the following additional insect species: *Leptinotarsa decemlineata* (Colorado potato beetle, hereinafter referred to as *Leptinotarsa*), *Tribolium castaneum* (flour beetle, hereinafter referred to as *Tribolium*), and *Heliothis virescens* (tobacco budworm, hereinafter referred to as *Heliothis*).

25

The newly identified insect p53 nucleic acids can be used for the generation of mutant phenotypes in animal models or in living cells that can be used to study regulation of p53, and the use of p53 as a drug or pesticide target. Due to the ability to rapidly carry out large-scale, systematic genetic screens, the use of invertebrate model organisms such as *Drosophila* has great utility for analyzing the expression and mis-expression of p53 protein. Thus, the invention provides a superior approach for identifying other components involved

30

in the synthesis, activity, and regulation of p53 proteins. Systematic genetic analysis of p53 using invertebrate model organisms can lead to the identification and validation of compound targets directed to components of the p53 pathway. Model organisms or cultured cells that have been genetically engineered to express p53 can be used to screen candidate compounds for their ability to modulate p53 expression or activity, and thus are useful in the identification of new drug targets, therapeutic agents, diagnostics and prognostics useful in the treatment of disorders associated with cell cycle, DNA repair, and apoptosis. The details of the conditions used for the identification and/or isolation of insect p53 nucleic acids and proteins are described in the Examples section below. Various non-limiting embodiments of the invention, applications and uses of the insect p53 genes and proteins are discussed in the following sections. The entire contents of all references, including patent applications, cited herein are incorporated by reference in their entireties for all purposes. Additionally, the citation of a reference in the preceding background section is not an admission of prior art against the claims appended hereto.

p53 Nucleic Acids

The following nucleic acid sequences encoding insect p53 are described herein: SEQ ID NO:1, isolated from *Drosophila*, and referred to herein as DMp53; SEQ ID NO:3, isolated from *Leptinotarsa*, and referred to herein as CPBp53; SEQ ID NO:5 and SEQ ID NO:7, isolated from *Tribolium*, and referred to herein as TRIB-Ap53 and TRIB-Bp53, respectively; and SEQ ID NO:9, isolated from *Heliothis*, and referred to herein as HELIOp53. The genomic sequence of the DMp53 gene is provided in SEQ ID NO:18.

In addition to the fragments and derivatives of SEQ ID NOs:1, 3, 5, 7, 9, and 18, as described in detail below, the invention includes the reverse complements thereof. Also, the subject nucleic acid sequences, derivatives and fragments thereof may be RNA molecules comprising the nucleotide sequences of SEQ ID NOs:1, 3, 5, 7, 9, and 18 (or derivative or fragment thereof) wherein the base U (uracil) is substituted for the base T (thymine). The DNA and RNA sequences of the invention can be single- or double-stranded. Thus, the term "isolated nucleic acid sequence" or "isolated nucleic acid molecule", as used herein, includes the reverse complement, RNA equivalent, DNA or RNA single- or double-stranded sequences, and DNA/RNA hybrids of the sequence being described, unless otherwise indicated.

Fragments of the p53 nucleic acid sequences can be used for a variety of purposes. Interfering RNA (RNAi) fragments, particularly double-stranded (ds) RNAi, can be used to

generate loss-of-function phenotypes. p53 nucleic acid fragments are also useful as nucleic acid hybridization probes and replication/amplification primers. Certain "antisense" fragments, i.e. that are reverse complements of portions of the coding sequence of any of SEQ ID NO:1, 3, 5, 7, 9, or 18 have utility in inhibiting the function of p53 proteins. The fragments are of length sufficient to specifically hybridize with the corresponding SEQ ID NO:1, 3, 5, 7, 9, or 18. The fragments consist of or comprise at least 12, preferably at least 24, more preferably at least 36, and more preferably at least 96 contiguous nucleotides of any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18. When the fragments are flanked by other nucleic acid sequences, the total length of the combined nucleic acid sequence is less than 15 kb, preferably less than 10 kb or less than 5kb, more preferably less than 2 kb, and in some cases, preferably less than 500 bases. Preferred p53 nucleic acid fragments comprise regulatory elements that may reside in the 5' UTR and/or encode one or more of the following domains: an activation domain, a DNA binding domain, a linker domain, an oligomerization domain, and a basic regulatory domain. The approximate locations of these regions in SEQ ID Nos 1, 3, and 5, and in the corresponding amino acid sequences of SEQ ID Nos 2, 4, and 6, 8, are provided in Table 1.

TABLE 1

	SEQ ID NOs		
	1/2	3/4	5/6
Insect Genus	<i>Drosophila</i>	<i>Leptinotarsa</i>	<i>Tribolium</i>
5' UTR	na 1-111	na 1-120	na 1-93
Activation Domain	na 112-257 aa 1-48	na 121-300 aa 1-60	na 94-277 aa 1-60
DNA Binding Domain	na 366-954 aa 85-280	na 321-936 aa 67-271	na 280-892 aa 62-265
Linker Domain	na 999-1056 aa 296-314	na 937-999 aa 272-292	na 893-958 aa 266-287
Oligomerization Domain	na 1065-1170 aa 318-352	na 1000-1113 aa 293-330	na 959-1075 aa 288-326
Basic Regulatory Domain	na 1179-1269 aa 356-385	na 1114-1182 aa 331-353	na 1076-1147 aa 327-350

Further preferred are fragments of bases 354-495 of SEQ ID NO:7 and bases 315-414 of SEQ ID NO:9 of at least 12, preferably at least 24, more preferably at least 36, and most preferably at least 96 contiguous nucleotides.

The subject nucleic acid sequences may consist solely of any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or fragments thereof. Alternatively, the subject nucleic acid sequences and fragments thereof may be joined to other components such as labels, peptides, agents that facilitate transport across cell membranes, hybridization-triggered cleavage agents or
5 intercalating agents. The subject nucleic acid sequences and fragments thereof may also be joined to other nucleic acid sequences (i.e. they may comprise part of larger sequences) and are of synthetic/non-natural sequences and/or are isolated and/or are purified, i.e. unaccompanied by at least some of the material with which it is associated in its natural state. Preferably, the isolated nucleic acids constitute at least about 0.5%, and more
10 preferably at least about 5% by weight of the total nucleic acid present in a given fraction, and are preferably recombinant, meaning that they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome.

Derivative nucleic acid sequences of p53 include sequences that hybridize to the
15 nucleic acid sequence of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringency conditions such that the hybridizing derivative nucleic acid is related to the subject nucleic acid by a certain degree of sequence identity. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule. Stringency of
20 hybridization refers to conditions under which nucleic acids are hybridizable. The degree of stringency can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. As used herein, the term "stringent hybridization conditions" are those normally used by one of skill in the art to establish at least about a 90% sequence identity between complementary pieces of
25 DNA or DNA and RNA. "Moderately stringent hybridization conditions" are used to find derivatives having at least about a 70% sequence identity. Finally, "low-stringency hybridization conditions" are used to isolate derivative nucleic acid molecules that share at least about 50% sequence identity with the subject nucleic acid sequence.

The ultimate hybridization stringency reflects both the actual hybridization
30 conditions as well as the washing conditions following the hybridization, and it is well known in the art how to vary the conditions to obtain the desired result. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). A preferred derivative nucleic acid is

capable of hybridizing to any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1 h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

Derivative nucleic acid sequences that have at least about 70% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 are capable of hybridizing to any one of SEQ ID NO:1, 3, 5, 7, 9, and 18 under moderately stringent conditions that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Other preferred derivative nucleic acid sequences are capable of hybridizing to any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 under low stringency conditions that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

As used herein, "percent (%) nucleic acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides in the candidate derivative nucleic acid sequence identical with the nucleotides in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410; <http://blast.wustl.edu/blast/README.html>; hereinafter referred to generally as "BLAST") with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database

against which the sequence of interest is being searched. A percent (%) nucleic acid sequence identity value is determined by the number of matching identical nucleotides divided by the sequence length for which the percent identity is being reported.

Derivative p53 nucleic acid sequences usually have at least 50% sequence identity, preferably at least 60%, 70%, or 80% sequence identity, more preferably at least 85% sequence identity, still more preferably at least 90% sequence identity, and most preferably at least 95% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or domain-encoding regions thereof.

In one preferred embodiment, the derivative nucleic acid encodes a polypeptide comprising a p53 amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, or 10, or a fragment or derivative thereof as described further below under the subheading "p53 proteins". A derivative p53 nucleic acid sequence, or fragment thereof, may comprise 100% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, but be a derivative thereof in the sense that it has one or more modifications at the base or sugar moiety, or phosphate backbone. Examples of modifications are well known in the art (Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such derivatives may be used to provide modified stability or any other desired property.

Another type of derivative of the subject nucleic acid sequences includes corresponding humanized sequences. A humanized nucleic acid sequence is one in which one or more codons has been substituted with a codon that is more commonly used in human genes. Preferably, a sufficient number of codons have been substituted such that a higher level expression is achieved in mammalian cells than what would otherwise be achieved without the substitutions. The following list shows, for each amino acid, the calculated codon frequency (number in parentheses) in humans genes for 1000 codons (Wada *et al.*, Nucleic Acids Research (1990) 18(Suppl.):2367-2411):

Human codon frequency per 1000 codons:

ARG: CGA (5.4), CGC (11.3), CGG (10.4), CGU (4.7), AGA (9.9), AGG (11.1)
 LEU: CUA (6.2), CUC (19.9), CUG (42.5), CUU (10.7), UUA (5.3), UUG (11.0)
 SER: UCA (9.3), UCC (17.7), UCG (4.2), UCU (13.2), AGC (18.7), AGU (9.4)
 THR: ACA (14.4), ACC (23.0), ACG (6.7), ACU (12.7)
 PRO: CCA (14.6), CCC (20.0), CCG (6.6), CCU (15.5)
 ALA: GCA (14.0), GCC (29.1), GCG (7.2), GCU (19.6)
 GLY: GGA (17.1), GGC (25.4), GGG (17.3), GGU (11.2)
 VAL: GUA (5.9), GUC (16.3), GUG (30.9), GUU (10.4)
 LYS: AAA (22.2), AAG (34.9)
 ASN: AAC (22.6), AAU (16.6)
 GLN: CAA (11.1), CAG (33.6)

HIS: CAC (14.2), CAU (9.3)
 GLU: GAA (26.8), GAG (41.4)
 ASP: GAC (29.0), GAU (21.7)
 TYR: UAC (18.8), UAU (12.5)
 5 CYS: UGC (14.5), UGU (9.9)
 PHE: UUU (22.6), UUC (15.8)
 ILE: AUA (5.8), AUC (24.3), AUU (14.9)
 MET: AUG (22.3)
 TRP: UGG (13.8)
 10 TER: UAA (0.7), AUG (0.5), UGA (1.2)

Thus, a p53 nucleic acid sequence in which the glutamic acid codon, GAA has been replaced with the codon GAG, which is more commonly used in human genes, is an example of a humanized p53 nucleic acid sequence. A detailed discussion of the humanization of nucleic acid sequences is provided in U.S. Pat. No. 5,874,304 to Zolotukhin *et al.* Similarly, other nucleic acid derivatives can be generated with codon usage optimized for expression in other organisms, such as yeasts, bacteria, and plants, where it is desired to engineer the expression of p53 proteins by using specific codons chosen according to the preferred codons used in highly expressed genes in each organism.
 15
 20 More specific embodiments of preferred p53 proteins, fragments, and derivatives are discussed further below in connection under the subheading "p53 proteins".

Nucleic acid encoding the amino acid sequence of any of SEQ ID NOs:2, 4, 6, 8, and 10, or fragment or derivative thereof, may be obtained from an appropriate cDNA library prepared from any eukaryotic species that encodes p53 proteins such as vertebrates, preferably mammalian (*e.g.* primate, porcine, bovine, feline, equine, and canine species, 25 *etc.*) and invertebrates, such as arthropods, particularly insects species (preferably *Drosophila*, *Tribolium*, *Leptinotarsa*, and *Heliothis*), *acarids*, *crustacea*, *molluscs*, *nematodes*, and other worms. An expression library can be constructed using known methods. For example, mRNA can be isolated to make cDNA which is ligated into a
 30 suitable expression vector for expression in a host cell into which it is introduced. Various screening assays can then be used to select for the gene or gene product (*e.g.* oligonucleotides of at least about 20 to 80 bases designed to identify the gene of interest, or labeled antibodies that specifically bind to the gene product). The gene and/or gene product can then be recovered from the host cell using known techniques.

35 Polymerase chain reaction (PCR) can also be used to isolate nucleic acids of the p53 genes where oligonucleotide primers representing fragmentary sequences of interest amplify RNA or DNA sequences from a source such as a genomic or cDNA library (as

described by Sambrook *et al.*, *supra*). Additionally, degenerate primers for amplifying homologs from any species of interest may be used. Once a PCR product of appropriate size and sequence is obtained, it may be cloned and sequenced by standard techniques, and utilized as a probe to isolate a complete cDNA or genomic clone.

5 Fragmentary sequences of p53 nucleic acids and derivatives may be synthesized by known methods. For example, oligonucleotides may be synthesized using an automated DNA synthesizer available from commercial suppliers (*e.g.* Biosearch, Novato, CA; Perkin-Elmer Applied Biosystems, Foster City, CA). Antisense RNA sequences can be produced intracellularly by transcription from an exogenous sequence, *e.g.* from vectors that contain
10 antisense p53 nucleic acid sequences. Newly generated sequences may be identified and isolated using standard methods.

 An isolated p53 nucleic acid sequence can be inserted into any appropriate cloning vector, for example bacteriophages such as lambda derivatives, or plasmids such as PBR322, pUC plasmid derivatives and the Bluescript vector (Stratagene, San Diego, CA).
15 Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, *etc.*, or into a transgenic animal such as a fly. The transformed cells can be cultured to generate large quantities of the p53 nucleic acid. Suitable methods for isolating and producing the subject nucleic acid sequences are well-known in the art (Sambrook *et al.*, *supra*; DNA Cloning: A Practical Approach, Vol. 1, 2, 3, 4, (1995)
20 Glover, ed., MRL Press, Ltd., Oxford, U.K.).

 The nucleotide sequence encoding a p53 protein or fragment or derivative thereof, can be inserted into any appropriate expression vector for the transcription and translation of the inserted protein-coding sequence. Alternatively, the necessary transcriptional and translational signals can be supplied by the native p53 gene and/or its flanking regions. A
25 variety of host-vector systems may be utilized to express the protein-coding sequence such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. If expression in plants is desired, a variety of transformation constructs, vectors and
30 methods are known in the art (see U.S. Pat. No. 6,002,068 for review). Expression of a p53 protein may be controlled by a suitable promoter/enhancer element. In addition, a host cell strain may be selected which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired

To detect expression of the p53 gene product, the expression vector can comprise a promoter operably linked to a p53 gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the p53 gene product based on the physical or functional properties of the p53 protein in *in vitro* assay systems (*e.g.* immunoassays or cell cycle assays). The p53 protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product as described above.

Once a recombinant that expresses the p53 gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). The amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant and can thus be synthesized by standard chemical methods (Hunkapiller *et al.*, Nature (1984) 310:105-111). Alternatively, native p53 proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification).

p33 and Rb Nucleic Acids

The invention also provides nucleic acid sequences for *Drosophila* p33 (SEQ ID NO:19), and Rb (SEQ ID NO:21) tumor suppressors. Derivatives and fragments of these sequences can be prepared as described above for the p53 sequences. Preferred fragments and derivatives comprise the same number of contiguous nucleotides or same degrees of percent identity as described above for p53 nucleic acid sequences. The disclosure below regarding various uses of p53 tumor suppressor nucleic acids and proteins (*e.g.* transgenic animals, tumor suppressor assays, *etc.*) also applies to the p33 and Rb tumor suppressor sequences disclosed herein.

p53 Proteins

The CLUSTALW program (Thompson, *et al.*, Nucleic Acids Research (1994) 22(22):4673-4680) was used to align the insect p53 proteins described herein with p53 proteins from human (Zakut-Houri *et al.*, EMBO J. (1985) 4:1251-1255; GenBank gi:129369), *Xenopus* (Sousi *et al.*, Oncogene (1987) 1:71-78; GenBank gi:129374), and squid (GenBank gi:1244762). The alignment generated is shown in Figure 1 and reveals a number of features in the insect p53 proteins that are characteristic of the previously-

identified p53 proteins. With respect to general areas of structural similarity, the DMp53, CPBp53, and TRIB-Ap53 proteins can be roughly divided into three regions: a central region which exhibits a high degree of sequence homology with other known p53 family proteins and which roughly corresponds to the DNA binding domain of this protein family (Cho *et al.*, Science (1994) 265:346-355), and flanking N-terminal and C-terminal regions which exhibit significantly less homology but which correspond in overall size to other p53 family proteins. The fragmentary polypeptide sequences encoded by the TRIB-Bp53 and HELIOp53 cDNAs are shown by the multiple sequence alignment to be derived from the central region – the conserved DNA-binding domain. Significantly, the protein sequence alignment allowed the assignment of the domains in the DMp53, CPBp53, and TRIB-A p53 proteins listed in Table 1 above, based on sequence homology with previously characterized domains of human p53 (Sousi and May, J. Mol Biol (1996) 260:623-637; Levine, *supra*; Prives, Cell (1998) 95:5-8).

Importantly, the most conserved central regions of the DMp53, CPBp53, and TRIB-A p53 proteins correspond almost precisely to the known functional boundaries of the DNA binding domain of human p53, indicating that these proteins are likely to exhibit similar DNA binding properties to those of human p53. A detailed examination of the conserved residues in this domain further emphasizes the likely structural and functional similarities between human p53 and the insect p53 proteins. First, residues of the human p53 known to be involved in direct DNA contacts (K120, S241, R248, R273, C277, and R280) correspond to identical or similar residues in the DMp53 protein (K113, S230, R234, K259, C263, and R266), and identical residues in the CPBp53 protein (K92, S216, R224, R249, C253, and R256), and the TRIB-Ap53 protein (K88, S213, R220, R245, C249, and R252). Also, with regard to the overall folding of this domain, it was notable that four key residues that coordinate the zinc ligand in the DNA binding domain of human p53 (C176, H179, C238, and C242) are precisely conserved in the DMp53 protein (C156, H159, C227, and C231), the CPBp53 protein (C147, H150, C213, and C217), and the TRIB-A p53 protein (C144, H147, C210, C214). Furthermore, it was striking that the mutational hot spots in human p53 most frequently altered in cancer (R175, G245, R248, R249, R273, and R282), are either identical or conserved amino acid residues in the corresponding positions of the DMp53 protein (R155, G233, R234, K235, K259, and R268), the CPBp53 protein (R146, G221, R224, R225, R249, and K258), and the TRIB-Ap53 protein (R143, G217, R220, R221, R245, and K254).

Interestingly, the insect p53s also have distinct differences from the Human, *Xenopus*, and squid p53s. Specifically, insect p53s contain a unique amino acid sequence within the DNA recognition domain that has the following sequence: (R or K)(I or V)C(S or T)CPKRD. Specifically, amino acid residues 259 to 267 of DMp53 have the sequence: KICTCPKRD; residues 249 to 257 of CPBp53 have the sequence: RICSCPKRD; and residues 245-253 of TRIB-Ap53 have the sequence: RVCSCPKRD. This is in distinct contrast to the Human, *Xenopus*, and squid p53s which have the following corresponding sequence: R(I or V)CACPGRD.

Another region of insect p53s that distinctly differs from previously identified p53s lies in the zinc coordination region of the DNA binding domain. The following sequence is conserved within the insect p53s: FXC(K or Q)NSC (where X = any amino acid). Specifically, residues 225-231 of DMp53 have the sequence: FVCQNSC; residues 211-217 of CPBp53 and residues 208-214 of TRIB-Ap53 have the sequence FVCKNSC; and the corresponding residues in Helio-p53, as shown in Figure 1, have the sequence: FSCKNSC. In contrast, the corresponding sequence in Human and *Xenopus* p53 is YMCNSSC, and in squid it is FMCLGSC.

The high degree of structural homology in the presumptive DNA binding domain of the insect p53 proteins has important implications for engineering derivative (*e.g.* mutant) forms of these p53 genes for tests of function *in vitro* and *in vivo*, and for genetic dissection or manipulation of the p53 pathway in transgenic insects or insect cell lines. Dominant negative forms of human p53 have been generated by creating altered proteins which have a defective DNA binding domain, but which retain a functional oligomerization domain (Brachman *et al.*, Proc Natl Acad Sci USA (1996) 93:4091-4095). Such dominant negative mutant forms are extremely useful for determining the effects of loss-of-function of p53 in assays of interest. Thus, mutations in highly conserved positions within the DNA binding domain of the insect p53 proteins, which correspond to residues known to be important for the structure and function of human p53 (such as R175H, H179N, and R280T of human p53), are likely to result in dominant negative forms of insect p53 proteins. For example, specific mutations in the DMp53 protein to create dominant negative mutant forms of the protein include R155H, H159N, and R266T and for the TRIB-A p53 protein include R143H, H147N, and R252T.

Although other domains of the insect p53 proteins, aside from the DNA binding domain, exhibit significantly less homology compared to the known p53 family proteins, the sequence alignment provides important information about their structure and potential

function. Notably, just as in the human p53 protein, the C-terminal 20-25 amino acids of the protein comprise a putative region that extends beyond the oligomerization domain, suggesting an analogous function for this region of the insect p53 proteins in regulating activity of the protein. Since deletion of the C-terminal regulatory domain in human p53
5 has been shown to generate constitutively activated forms of the protein (Hupp and Lane, Curr. Biol. (1994) 4:865-875), it is expected that removal of most or all of the corresponding regulatory domain from the insect p53 proteins will generate an activated protein form. Thus preferred truncated forms of the insect p53 proteins lack at least 10 C-terminal amino acids, more preferably at least 15 amino acids, and most preferably at least
10 20 C-terminal amino acids. For example, a preferred truncated version of DMp53 comprises amino acid residues 1-376, more preferably residues 1-371, and most preferably residues 1-366 of SEQ ID NO:2. Such constitutively activated mutant forms of the protein are very useful for tests of protein function using *in vivo* and *in vitro* assays, as well as for genetic analysis.

15 The oligomerization domain of the insect p53 proteins exhibit very limited skeletal sequence homology with other p53 family proteins, although the length of this region is similar to that of other p53 family proteins. The extent of sequence divergence in this region of the insect proteins raises the possibility that the insect p53 protein may be unable to form hetero-oligomers with p53 proteins from vertebrates or squid. And, although the
20 linker domain located between the DNA binding and oligomerization domains also exhibits relatively little sequence conservation, this region of any of the DMp53, CPBp53, and TRIB-A p53 proteins contains predicted nuclear localization signals similar to those identified in human p53 (Shaulsky *et al.*, Mol Cell Biol (1990) 10:6565-6577).

The activation domain at the N-terminus of the insect p53 proteins also exhibits
25 little sequence identity with other p53 family proteins, although the size of this region is roughly the same as that of human p53. Nonetheless, an important feature of this domain is the relative concentration of acidic residues in the insect p53 proteins. Consequently, it is likely that this N-terminal domain of any of the DMp53, CPBp53, and TRIB-Ap53 proteins will similarly exert the functional activity of a transcriptional activation domain to that of
30 the human p53 domain (Thut *et al.*, Science (1995) 267:100-104). Interestingly, the DMp53, CPBp53 and TRIB-A p53 proteins do not appear to possess a highly conserved sequence motif, FxxLWxxL, found at the N-terminus of vertebrate and squid p53 family proteins. In the human p53 gene, these conserved residues in this motif participate in a

specific interaction between human p53 proteins and mdm2 (Kussie *et al.*, Science (1996) 274:948-953).

It is important to note that, although there is no sequence similarity between the insect p53s and other p53 family members in the C- and N-termini, these regions of p53 contain secondary structure characteristic of p53-related proteins. For example, the human p53 binds DNA as a homo-tetramer and self-association is mediated by a β -sheet and amphipathic α -helix located in the C-terminus of the protein. A similar β -sheet-turn- α -helix is predicted in the C-terminus of DMp53. Further, the N-terminus of the human p53 is a region that includes a transactivation domain and residues critical for binding to the mdm-2 protein. The N-terminus of the DMp53 also include acidic amino acids and likely functions as a transactivation domain.

p53 proteins of the invention comprise or consist of an amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments or derivatives thereof. Compositions comprising these proteins may consist essentially of the p53 protein, fragments, or derivatives, or may comprise additional components (*e.g.* pharmaceutically acceptable carriers or excipients, culture media, *etc.*). p53 protein derivatives typically share a certain degree of sequence identity or sequence similarity with any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments thereof. As used herein, "percent (%) amino acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of amino acids in the candidate derivative amino acid sequence identical with the amino acid in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by BLAST (Altschul *et al.*, *supra*) using the same parameters discussed above for derivative nucleic acid sequences. A % amino acid sequence identity value is determined by the number of matching identical amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation. A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine;

interchangeable basic amino acids arginine, lysine and histidine; interchangeable acidic amino acids aspartic acid and glutamic acid; and interchangeable small amino acids alanine, serine, cystine, threonine, and glycine.

In one preferred embodiment, a p53 protein derivative shares at least 50% sequence identity or similarity, preferably at least 60%, 70%, or 80% sequence identity or similarity, more preferably at least 85% sequence similarity or identity, still more preferably at least 90% sequence similarity or identity, and most preferably at least 95% sequence identity or similarity with a contiguous stretch of at least 10 amino acids, preferably at least 25 amino acids, more preferably at least 40 amino acids, still more preferably at least 50 amino acids, more preferably at least 100 amino acids, and in some cases, the entire length of any one of SEQ ID NOs: 2, 4, 6, 8, or 10. Further preferred derivatives share these % sequence identities with the domains of SEQ ID NOs 2, 4 and 6 listed in Table I above. Additional preferred derivatives comprise a sequence that shares 100% similarity with any contiguous stretch of at least 10 amino acids, preferably at least 12, more preferably at least 15, and most preferably at least 20 amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof. Further preferred fragments comprise at least 7 contiguous amino acids, preferably at least 9, more preferably at least 12, and most preferably at least 17 contiguous amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof.

Other preferred p53 polypeptides, fragments or derivatives consist of or comprise a sequence selected from the group consisting of RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, and KVCTCPGRD (i.e. sequences of the formula: (R or K)(I or V)C(S or T)CPGRD). Additional preferred p53 polypeptides, fragments or derivatives, consist of or comprise a sequence selected from the group consisting of FXCKNSC and FXCQNSC, where X = any amino acid.

The fragment or derivative of any of the p53 proteins is preferably "functionally active" meaning that the p53 protein derivative or fragment exhibits one or more functional activities associated with a full-length, wild-type p53 protein comprising the amino acid sequence of any of SEQ ID NOs: 2, 4, 6, 8, or 10. As one example, a fragment or derivative may have antigenicity such that it can be used in immunoassays, for immunization, for inhibition of p53 activity, *etc.*, as discussed further below regarding generation of antibodies to p53 proteins. Preferably, a functionally active p53 fragment or derivative is one that displays one or more biological activities associated with p53 proteins such as regulation of

the cell cycle, or transcription control. The functional activity of p53 proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey). Example 12 below describes a variety of suitable assays for assessing p53 function.

P 53 derivatives can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned p53 gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) (Wells *et al.*, Philos. Trans. R. Soc. London SerA (1986) 317:415), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*, and expressed to produce the desired derivative. Alternatively, a p53 gene can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. A variety of mutagenesis techniques are known in the art such as chemical mutagenesis, *in vitro* site-directed mutagenesis (Carter *et al.*, Nucl. Acids Res. (1986) 13:4331), use of TAB[®] linkers (available from Pharmacia and Upjohn, Kalamazoo, MI), *etc.*

At the protein level, manipulations include post translational modification, *e.g.* glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications may be carried out by known technique (*e.g.* specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*). Derivative proteins can also be chemically synthesized by use of a peptide synthesizer, for example to introduce nonclassical amino acids or chemical amino acid analogs as substitutions or additions into the p53 protein sequence.

Chimeric or fusion proteins can be made comprising a p53 protein or fragment thereof (preferably comprising one or more structural or functional domains of the p53 protein) joined at its N- or C-terminus via a peptide bond to an amino acid sequence of a different protein. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other in the proper coding frame using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer.

p33 and Rb Proteins

The invention also provides amino acid sequences for *Drosophila* p33 (SEQ ID NO:20), and Rb (SEQ ID NO:22) tumor suppressors. Derivatives and fragments of these sequences can be prepared as described above for the p53 protein sequences. Preferred fragments and derivatives comprise the same number of contiguous amino acids or same degrees of percent identity or similarity as described above for p53 amino acid sequences.

p53 Gene Regulatory Elements

p53 gene regulatory DNA elements, such as enhancers or promoters that reside within the 5' UTRs of SEQ ID NOs 1, 3, and 5, as shown in Table I above, or within nucleotides 1-1225 of SEQ ID NO:18, can be used to identify tissues, cells, genes and factors that specifically control p53 protein production. Preferably at least 20, more preferably at least 25, and most preferably at least 50 contiguous nucleotides within the 5' UTRs are used. Analyzing components that are specific to p53 protein function can lead to an understanding of how to manipulate these regulatory processes, for either pesticide or therapeutic applications, as well as an understanding of how to diagnose dysfunction in these processes.

Gene fusions with the p53 regulatory elements can be made. For compact genes that have relatively few and small intervening sequences, such as those described herein for *Drosophila*, it is typically the case that the regulatory elements that control spatial and temporal expression patterns are found in the DNA immediately upstream of the coding region, extending to the nearest neighboring gene. Regulatory regions can be used to construct gene fusions where the regulatory DNAs are operably fused to a coding region for a reporter protein whose expression is easily detected, and these constructs are introduced as transgenes into the animal of choice. An entire regulatory DNA region can be used, or the regulatory region can be divided into smaller segments to identify sub-elements that might be specific for controlling expression a given cell type or stage of development. One suitable method to decipher regions containing regulatory sequences is by an *in vitro* CAT assay (Mercer, Crit. Rev. Euk. Gene Exp. (1992) 2:251-263; Sambrook *et al.*, *supra*; and Gorman *et al.*, Mol. Cell. Biol. (1992) 2:1044-1051). Additional reporter proteins that can be used for construction of these gene fusions include *E. coli* beta-galactosidase and green fluorescent protein (GFP). These can be detected readily *in situ*, and thus are useful for histological studies and can be used to sort cells that express p53 proteins (O'Kane and

Gehring PNAS (1987) 84(24):9123-9127; Chalfie *et al.*, Science (1994) 263:802-805; and Cumberledge and Krasnow (1994) Methods in Cell Biology 44:143-159). Recombinase proteins, such as FLP or cre, can be used in controlling gene expression through site-specific recombination (Golic and Lindquist (1989) Cell 59(3):499-509; White *et al.*,
5 Science (1996) 271:805-807). Toxic proteins such as the reaper and hid cell death proteins, are useful to specifically ablate cells that normally express p53 proteins in order to assess the physiological function of the cells (Kingston. In Current Protocols in Molecular Biology (1998) Ausubel *et al.*, John Wiley & Sons, Inc. sections 12.0.3-12.10) or any other protein where it is desired to examine the function this particular protein specifically in cells that
10 synthesize p53 proteins.

Alternatively, a binary reporter system can be used, similar to that described further below, where the p53 regulatory element is operably fused to the coding region of an exogenous transcriptional activator protein, such as the GAL4 or tTA activators described below, to create a p53 regulatory element "driver gene". For the other half of the binary
15 system the exogenous activator controls a separate "target gene" containing a coding region of a reporter protein operably fused to a cognate regulatory element for the exogenous activator protein, such as UAS_G or a tTA-response element, respectively. An advantage of a binary system is that a single driver gene construct can be used to activate transcription from preconstructed target genes encoding different reporter proteins, each with its own
20 uses as delineated above.

p53 regulatory element-reporter gene fusions are also useful for tests of genetic interactions, where the objective is to identify those genes that have a specific role in controlling the expression of p53 genes, or promoting the growth and differentiation of the tissues that expresses the p53 protein. p53 gene regulatory DNA elements are also useful in
25 protein-DNA binding assays to identify gene regulatory proteins that control the expression of p53 genes. The gene regulatory proteins can be detected using a variety of methods that probe specific protein-DNA interactions well known to those skilled in the art (Kingston, *supra*) including *in vivo* footprinting assays based on protection of DNA sequences from chemical and enzymatic modification within living or permeabilized cells; and *in vitro*
30 footprinting assays based on protection of DNA sequences from chemical or enzymatic modification using protein extracts, nitrocellulose filter-binding assays and gel electrophoresis mobility shift assays using radioactively labeled regulatory DNA elements mixed with protein extracts. Candidate p53 gene regulatory proteins can be purified using a combination of conventional and DNA-affinity purification techniques. Molecular cloning

strategies can also be used to identify proteins that specifically bind p53 gene regulatory DNA elements. For example, a *Drosophila* cDNA library in an expression vector, can be screened for cDNAs that encode p53 gene regulatory element DNA-binding activity. Similarly, the yeast "one-hybrid" system can be used (Li and Herskowitz. *Science* (1993) 262:1870-1874; Luo *et al.*, *Biotechniques* (1996) 20(4):564-568; Vidal *et al.*, *PNAS* (1996) 93(19):10315-10320).

Assays for tumor suppressor genes

The p53 tumor suppressor gene encodes a transcription factor implicated in regulation of cell proliferation, control of the cell cycle, and induction of apoptosis. Various experimental methods may be used to assess the role of the insect p53 genes in each of these areas.

Transcription activity assays

Due to its acidic region, wild type p53 binds both specifically and non-specifically to DNA in order to mediate its function (Zambetti and Levine, *supra*). Transcriptional regulation by the p53 protein or its fragments may be examined by any method known in the art. An electrophoretic mobility shift assay can be used to characterize DNA sequences to which p53 binds, and thus can assist in the identification of genes regulated by p53. Briefly, cells are grown and transfected with various amounts of wild type or mutated transcription factor of interest (in this case, p53), harvested 48 hr after transfection, and lysed to prepare nuclear extracts. Preparations of *Drosophila* nuclear extracts for use in mobility shift assays may be done as described in Dignam *et al.*, *Nucleic Acids Res.* (1983) 11:1475-1489. Additionally, complementary, single-stranded oligonucleotides corresponding to target sequences for binding are synthesized and self-annealed to a final concentration of 10-15 ng/ μ l. Double stranded DNA is verified by gel electrophoretic analysis (*e.g.*, on a 7% polyacrylamide gel, by methods known in the art), and end-labeled with 20 μ Ci [32P] γ -dATP. The nuclear extracts are mixed with the double stranded target sequences under conditions conducive for binding and the results are analyzed by polyacrylamide gel electrophoresis.

Another suitable method to determine DNA sequences to which p53 binds is by DNA footprinting (Schmitz *et al.*, *Nucleic Acids Research* (1978) 5:3157-3170).

Apoptosis assays

A variety of methods may be used to examine apoptosis. One method is the terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling

(TUNEL) assay which measures the nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, Nature (1994) 371:346-347; White *et al.*, Science (1994) 264:677-683). Additionally, commercial kits can be used for detection of apoptosis (ApoAlert® available from Clontech (Palo Alto, CA)).

5 Apoptosis may also be assayed by a variety of staining methods. Acridine orange can be used to detect apoptosis in cultured cells (Lucas *et al.*, Blood (1998) 15:4730-41) and in intact *Drosophila* tissues, which can also be stained with Nile Blue (Abrams *et al.*, Development (1993) 117:29-43). Another assay that can be used to detect DNA laddering employs ethidium bromide staining and electrophoresis of DNA on an agarose gel (Civielli
10 *et al.*, Int. J. Cancer (1995) 27:673-679; Young, J. Biol. Chem. (1998) 273:25198-25202).

Proliferation and cell cycle assays

Proliferating cells may be identified by bromodeoxyuridine (BRDU) incorporation into cells undergoing DNA synthesis and detection by an anti-BRDU antibody (Hoshino *et al.*, Int. J. Cancer (1986) 38:369; Campana *et al.*, J. Immunol. Meth. (1988) 107:79). This
15 assay can be used to reproducibly identify S-phase cells in *Drosophila* embryos (Edgar and O'Farrell, Cell (1990) 62:469-480) and imaginal discs (Secombe *et al.*, Genetics (1998) 149:1867-1882). S-phase DNA syntheses can also be quantified by measuring [³H]-thymidine incorporation using a scintillation counter (Chen, Oncogene (1996) 13:1395-403; Jeoung, J. Biol. Chem. (1995) 270:18367-73). Cell proliferation may be measured by
20 counting samples of a cell population over time, for example using a hemacytometer and Trypan-blue staining.

The DNA content and/or mitotic index of the cells may be measured based on the DNA ploidy value of the cell using a variety of methods known in the art such as a propidium iodide assay (Turner *et al.*, Prostate (1998) 34:175-81) or Feulgen staining using
25 a computerized microdensitometry staining system (Bacus, Am. J. Pathol. (1989) 135:783-92).

The effect of p53 overexpression or loss-of-function on *Drosophila* cell proliferation can be assayed *in vivo* using an assay in which clones of cells with altered gene expression are generated in the developing wing disc of *Drosophila* (Neufeld *et al.*, Cell (1998)
30 93:1183-93). The clones coexpress GFP, which allows the size and DNA content of the mutant and wild-type cells from dissociated discs to be compared by FACS analysis.

Tumor formation and transformation assays

A variety of *in vivo* and *in vitro* tumor formation assays are known in the art that can be used to assay p53 function. Such assays can be used to detect foci formation (Beenken,

J. Surg. Res. (1992) 52:401-5), *in vitro* transformation (Ginsberg, Oncogene. (1991) 6:669-72), tumor formation in nude mice (Endlich, Int. J. Radiat. Biol. (1993) 64:715-26), tumor formation in *Drosophila* (Tao *et al.*, Nat. Genet. (1999) 21:177-181), and anchorage-independent growth in soft agar (Endlich, *supra*). Loss of indicia of differentiation may be indicate transformation, including loss of differentiation markers, cell rounding, loss of adhesion, loss of polarity, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, and expression of fetal antigens.

10 **Generation and Genetic Analysis of Animals and Cell Lines with Altered Expression of p53 Gene**

Both genetically modified animal models (i.e. *in vivo* models), such as *C. elegans* and *Drosophila*, and *in vitro* models such as genetically engineered cell lines expressing or mis-expressing p53 genes, are useful for the functional analysis of these proteins. Model systems that display detectable phenotypes, can be used for the identification and characterization of p53 genes or other genes of interest and/or phenotypes associated with the mutation or mis-expression of p53. The term "mis-expression" as used herein encompasses mis-expression due to gene mutations. Thus, a mis-expressed p53 protein may be one having an amino acid sequence that differs from wild-type (i.e. it is a derivative of the normal protein). A mis-expressed p53 protein may also be one in which one or more N- or C- terminal amino acids have been deleted, and thus is a "fragment" of the normal protein. As used herein, "mis-expression" also includes ectopic expression (*e.g.* by altering the normal spatial or temporal expression), over-expression (*e.g.* by multiple gene copies), underexpression, non-expression (*e.g.* by gene knockout or blocking expression that would otherwise normally occur), and further, expression in ectopic tissues.

The *in vivo* and *in vitro* models may be genetically engineered or modified so that they 1) have deletions and/or insertions of a p53 genes, 2) harbor interfering RNA sequences derived from a p53 gene, 3) have had an endogenous p53 gene mutated (*e.g.* contain deletions, insertions, rearrangements, or point mutations in the p53 gene), and/or 4) contain transgenes for mis-expression of wild-type or mutant forms of a p53 gene. Such genetically modified *in vivo* and *in vitro* models are useful for identification of genes and proteins that are involved in the synthesis, activation, control, *etc.* of p53, and also downstream effectors of p53 function, genes regulated by p53, *etc.* The model systems can be used for testing potential pharmaceutical and pesticidal compounds that interact with

p53, for example by administering the compound to the model system using any suitable method (*e.g.* direct contact, ingestion, injection, *etc.*) and observing any changes in phenotype, for example defective movement, lethality, *etc.* Various genetic engineering and expression modification methods which can be used are well-known in the art, including chemical mutagenesis, transposon mutagenesis, antisense RNAi, dsRNAi, and transgene-mediated mis-expression.

Generating Loss-of-function Mutations by Mutagenesis

Loss-of-function mutations in an insect p53 gene can be generated by any of several mutagenesis methods known in the art (Ashburner, In *Drosophila melanogaster*: A Laboratory Manual (1989), Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press: pp. 299-418; Fly pushing: The Theory and Practice of *Drosophila melanogaster* Genetics (1997) Cold Spring Harbor Press, Plainview, NY, hereinafter "Fly Pushing"). Techniques for producing mutations in a gene or genome include use of radiation (*e.g.*, X-ray, UV, or gamma ray); chemicals (*e.g.*, EMS, MMS, ENU, formaldehyde, *etc.*); and insertional mutagenesis by mobile elements including dysgenesis induced by transposon insertions, or transposon-mediated deletions, for example, male recombination, as described below. Other methods of altering expression of genes include use of transposons (*e.g.*, P element, EP-type "overexpression trap" element, mariner element, *piggyBac* transposon, hermes, minos, sleeping beauty, *etc.*) to misexpress genes; antisense; double-stranded RNA interference; peptide and RNA aptamers; directed deletions; homologous recombination; dominant negative alleles; and intrabodies.

Transposon insertions lying adjacent to a p53 gene can be used to generate deletions of flanking genomic DNA, which if induced in the germline, are stably propagated in subsequent generations. The utility of this technique in generating deletions has been demonstrated and is well-known in the art. One version of the technique using collections of P element transposon induced recessive lethal mutations (P lethals) is particularly suitable for rapid identification of novel, essential genes in *Drosophila* (Cooley *et al.*, Science (1988) 239:1121-1128; Spralding *et al.*, PNAS (1995) 92:10824-10830). Since the sequence of the P elements are known, the genomic sequence flanking each transposon insert is determined either by plasmid rescue (Hamilton *et al.*, PNAS (1991) 88:2731-2735) or by inverse polymerase chain reaction (Rehm, <http://www.fruitfly.org/methods/>). A more recent version of the transposon insertion technique in male *Drosophila* using P elements is known as P-mediated male recombination (Preston and Engels, Genetics (1996) 144:1611-1638).

Generating Loss-of-function Phenotypes Using RNA-based Methods

p53 genes may be identified and/or characterized by generating loss-of-function phenotypes in animals of interest through RNA-based methods, such as antisense RNA (Schubiger and Edgar, *Methods in Cell Biology* (1994) 44:697-713). One form of the
5 antisense RNA method involves the injection of embryos with an antisense RNA that is partially homologous to the gene of interest (in this case the p53 gene). Another form of the antisense RNA method involves expression of an antisense RNA partially homologous to the gene of interest by operably joining a portion of the gene of interest in the antisense orientation to a powerful promoter that can drive the expression of large quantities of
10 antisense RNA, either generally throughout the animal or in specific tissues. Antisense RNA-generated loss-of-function phenotypes have been reported previously for several *Drosophila* genes including cactus, pecanex, and Krüppel (LaBonne *et al.*, *Dev. Biol.* (1989) 136(1):1-16; Schuh and Jackle, *Genome* (1989) 31(1):422-425; Geisler *et al.*, *Cell* (1992) 71(4):613-621).

15 Loss-of-function phenotypes can also be generated by cosuppression methods (Bingham, *Cell* (1997) 90(3):385-387; Smyth, *Curr. Biol.* (1997) 7(12):793-795; Que and Jorgensen, *Dev. Genet.* (1998) 22(1):100-109). Cosuppression is a phenomenon of reduced gene expression produced by expression or injection of a sense strand RNA corresponding to a partial segment of the gene of interest. Cosuppression effects have been employed
20 extensively in plants and *C. elegans* to generate loss-of-function phenotypes. Cosuppression in *Drosophila* has been shown, where reduced expression of the Adh gene was induced from a white-Adh transgene (Pal-Bhadra *et al.*, *Cell* (1997) 90(3):479-490).

Another method for generating loss-of-function phenotypes is by double-stranded RNA interference (dsRNAi). This method is based on the interfering properties of double-
25 stranded RNA derived from the coding regions of gene, and has proven to be of great utility in genetic studies of *C. elegans* (Fire *et al.*, *Nature* (1998) 391:806-811), and can also be used to generate loss-of-function phenotypes in *Drosophila* (Kennerdell and Carthew, *Cell* (1998) 95:1017-1026; Misquitta and Patterson *PNAS* (1999) 96:1451-1456).

Complementary sense and antisense RNAs derived from a substantial portion of a gene of
30 interest, such as p53 gene, are synthesized *in vitro*, annealed in an injection buffer, and introduced into animals by injection or other suitable methods such as by feeding, soaking the animals in a buffer containing the RNA, *etc.* Progeny of the dsRNA treated animals are then inspected for phenotypes of interest (PCT publication no. WO99/32619).

dsRNAi can also be achieved by causing simultaneous expression *in vivo* of both sense and antisense RNA from appropriately positioned promoters operably fused to p53 sequences. Alternatively, the living food of an animal can be engineered to express sense and antisense RNA, and then fed to the animal. For example, *C. elegans* can be fed engineered *E. coli*, *Drosophila* can be fed engineered baker's yeast, and insects such as *Leptinotarsa* and *Heliothis* and other plant-eating animals can be fed transgenic plants engineered to produce the dsRNA.

RNAi has also been successfully used in cultured *Drosophila* cells to inhibit expression of targeted proteins (Dixon lab, University of Michigan, <http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html>). Thus, cell lines in culture can be manipulated using RNAi both to perturb and study the function of p53 pathway components and to validate the efficacy of therapeutic or pesticidal strategies which involve the manipulation of this pathway. A suitable protocol is described in Example 13.

15 **Generating Loss-of-function Phenotypes Using Peptide and RNA Aptamers**

Another method for generating loss-of-function phenotypes is by the use of peptide aptamers, which are peptides or small polypeptides that act as dominant inhibitors of protein function. Peptide aptamers specifically bind to target proteins, blocking their function ability (Kolonin and Finley, PNAS (1998) 95:14266-14271). Due to the highly selective nature of peptide aptamers, they may be used not only to target a specific protein, but also to target specific functions of a given protein (*e.g.* transcription function). Further, peptide aptamers may be expressed in a controlled fashion by use of promoters which regulate expression in a temporal, spatial or inducible manner. Peptide aptamers act dominantly; therefore, they can be used to analyze proteins for which loss-of-function mutants are not available.

Peptide aptamers that bind with high affinity and specificity to a target protein may be isolated by a variety of techniques known in the art. In one method, they are isolated from random peptide libraries by yeast two-hybrid screens (Xu *et al.*, PNAS (1997) 94:12473-12478). They can also be isolated from phage libraries (Hoogenboom *et al.*, Immunotechnology (1998) 4:1-20) or chemically generated peptides/libraries.

RNA aptamers are specific RNA ligands for proteins, that can specifically inhibit protein function of the gene (Good *et al.*, Gene Therapy (1997) 4:45-54; Ellington, *et al.*, Biotechnol. Annu. Rev. (1995) 1:185-214). *In vitro* selection methods can be used to identify RNA aptamers having a selected specificity (Bell *et al.*, J. Biol. Chem. (1998)

273:14309-14314). It has been demonstrated that RNA aptamers can inhibit protein function in *Drosophila* (Shi *et al.*, Proc. Natl. Acad. Sci USA (1999) 96:10033-10038). Accordingly, RNA aptamers can be used to decrease the expression of p53 protein or derivative thereof, or a protein that interacts with the p53 protein.

- 5 Transgenic animals can be generated to test peptide or RNA aptamers *in vivo* (Kolonin and Finley, *supra*). For example, transgenic *Drosophila* lines expressing the desired aptamers may be generated by P element mediated transformation (discussed below). The phenotypes of the progeny expressing the aptamers can then be characterized.

Generating Loss of Function Phenotypes Using Intrabodies

- 10 Intracellularly expressed antibodies, or intrabodies, are single-chain antibody molecules designed to specifically bind and inactivate target molecules inside cells. Intrabodies have been used in cell assays and in whole organisms such as *Drosophila* (Chen *et al.*, Hum. Gen. Ther. (1994) 5:595-601; Hassanzadeh *et al.*, Febs Lett. (1998) 16(1, 2):75-80 and 81-86). Inducible expression vectors can be constructed with intrabodies that
15 react specifically with p53 protein. These vectors can be introduced into model organisms and studied in the same manner as described above for aptamers.

Transgenesis

- Typically, transgenic animals are created that contain gene fusions of the coding regions of the p53 gene (from either genomic DNA or cDNA) or genes engineered to
20 encode antisense RNAs, cosuppression RNAs, interfering dsRNA, RNA aptamers, peptide aptamers, or intrabodies operably joined to a specific promoter and transcriptional enhancer whose regulation has been well characterized, preferably heterologous promoters/enhancers (i.e. promoters/enhancers that are non-native to the p53 genes being expressed).

- Methods are well known for incorporating exogenous nucleic acid sequences into
25 the genome of animals or cultured cells to create transgenic animals or recombinant cell lines. For invertebrate animal models, the most common methods involve the use of transposable elements. There are several suitable transposable elements that can be used to incorporate nucleic acid sequences into the genome of model organisms. Transposable elements are also particularly useful for inserting sequences into a gene of interest so that
30 the encoded protein is not properly expressed, creating a "knock-out" animal having a loss-of-function phenotype. Techniques are well-established for the use of P element in *Drosophila* (Rubin and Spradling, Science (1982) 218:348-53; U.S. Pat. No. 4,670,388). Additionally, transposable elements that function in a variety of species, have been

identified, such as PiggyBac (Thibault *et al.*, Insect Mol Biol (1999) 8(1):119-23), hobo, and hermes.

P elements, or marked P elements, are preferred for the isolation of loss-of-function mutations in *Drosophila* p53 genes because of the precise molecular mapping of these genes, depending on the availability and proximity of preexisting P element insertions for use as a localized transposon source (Hamilton and Zinn, Methods in Cell Biology (1994) 44:81-94; and Wolfner and Goldberg, Methods in Cell Biology (1994) 44:33-80). Typically, modified P elements are used which contain one or more elements that allow detection of animals containing the P element. Most often, marker genes are used that affect the eye color of *Drosophila*, such as derivatives of the *Drosophila* white or rosy genes (Rubin and Spradling, *supra*; and Klemenz *et al.*, Nucleic Acids Res. (1987) 15(10):3947-3959). However, in principle, any gene can be used as a marker that causes a reliable and easily scored phenotypic change in transgenic animals. Various other markers include bacterial plasmid sequences having selectable markers such as ampicillin resistance (Steller and Pirrotta, EMBO. J. (1985) 4:167-171); and lacZ sequences fused to a weak general promoter to detect the presence of enhancers with a developmental expression pattern of interest (Bellen *et al.*, Genes Dev. (1989) 3(9):1288-1300). Other examples of marked P elements useful for mutagenesis have been reported (Nucleic Acids Research (1998) 26:85-88; and <http://flybase.bio.indiana.edu>).

A preferred method of transposon mutagenesis in *Drosophila* employs the "local hopping" method (Tower *et al.* (Genetics (1993) 133:347-359). Each new P insertion line can be tested molecularly for transposition of the P element into the gene of interest (e.g. p53) by assays based on PCR. For each reaction, one PCR primer is used that is homologous to sequences contained within the P element and a second primer is homologous to the coding region or flanking regions of the gene of interest. Products of the PCR reactions are detected by agarose gel electrophoresis. The sizes of the resulting DNA fragments reveal the site of P element insertion relative to the gene of interest. Alternatively, Southern blotting and restriction mapping using DNA probes derived from genomic DNA or cDNAs of the gene of interest can be used to detect transposition events that rearrange the genomic DNA of the gene. P transposition events that map to the gene of interest can be assessed for phenotypic effects in heterozygous or homozygous mutant *Drosophila*.

In another embodiment, *Drosophila* lines carrying P insertions in the gene of interest, can be used to generate localized deletions using known methods (Kaiser.

Bioassays (1990) 12(6):297-301; Harnessing the power of *Drosophila* genetics, In *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology*, Goldstein and Fyrberg, Eds., Academic Press, Inc. San Diego, California). This is particularly useful if no P element transpositions are found that disrupt the gene of interest. Briefly, flies containing
5 P elements inserted near the gene of interest are exposed to a further round of transposase to induce excision of the element. Progeny in which the transposon has excised are typically identified by loss of the eye color marker associated with the transposable element. The resulting progeny will include flies with either precise or imprecise excision of the P element, where the imprecise excision events often result in deletion of genomic DNA
10 neighboring the site of P insertion. Such progeny are screened by molecular techniques to identify deletion events that remove genomic sequence from the gene of interest; and assessed for phenotypic effects in heterozygous and homozygous mutant *Drosophila*.

Recently a transgenesis system has been described that may have universal applicability in all eye-bearing animals and which has been proven effective in delivering
15 transgenes to diverse insect species (Berghammer *et al.*, Nature (1999) 402:370-371). This system includes: an artificial promoter active in eye tissue of all animal species, preferably containing three Pax6 binding sites positioned upstream of a TATA box (3xP3; Sheng *et al.* Genes Devel. (1997) 11:1122-1131); a strong and visually detectable marker gene, such as GFP or other autofluorescent protein genes (Pasher *et al.*, Gene (1992) 111:229-233;
20 U.S. Pat. No. 5,491,084); and promiscuous vectors capable of delivering transgenes to a broad range of animal species, for example transposon-based vectors derived from *Hermes*, *PiggyBac*, or *mariner*, or vectors based on pantropic VSV_G-pseudotyped retroviruses (Burns *et al.*, In Vitro Cell Dev Biol Anim (1996) 32:78-84; Jordan *et al.*, Insect Mol Biol (1998) 7: 215-222; US Pat. No. 5,670,345). Since the same transgenesis system can be
25 used in a variety of phylogenetically diverse animals, comparative functional studies are greatly facilitated, which is especially helpful in evaluating new applications to pest management.

In addition to creating loss-of-function phenotypes, transposable elements can be used to incorporate p53, or fragments or derivatives thereof, as an additional gene into any
30 region of an animal's genome resulting in mis-expression (including over-expression) of the gene. A preferred vector designed specifically for misexpression of genes in transgenic *Drosophila*, is derived from pGMR (Hay *et al.*, Development (1994) 120:2121-2129), is 9Kb long, and contains: an origin of replication for *E. coli*; an ampicillin resistance gene; P element transposon 3' and 5' ends to mobilize the inserted sequences; a White marker gene;

an expression unit comprising the TATA region of hsp70 enhancer and the 3'untranslated region of α -tubulin gene. The expression unit contains a first multiple cloning site (MCS) designed for insertion of an enhancer and a second MCS located 500 bases downstream, designed for the insertion of a gene of interest. As an alternative to transposable elements,
5 homologous recombination or gene targeting techniques can be used to substitute a heterologous p53 gene or fragment or derivative for one or both copies of the animal's homologous gene. The transgene can be under the regulation of either an exogenous or an endogenous promoter element, and be inserted as either a minigene or a large genomic fragment. Gene function can be analyzed by ectopic expression, using, for example,
10 *Drosophila* (Brand *et al.*, Methods in Cell Biology (1994) 44:635- 654).

Examples of well-characterized heterologous promoters that may be used to create transgenic *Drosophila* include heat shock promoters/enhancers such as the *hsp70* and *hsp83* genes. Eye tissue specific promoters/enhancers include *eyeless* (Mozer and Benzer, Development (1994) 120:1049-1058), *sevenless* (Bowtell *et al.*, PNAS (1991) 88(15):6853-
15 6857), and *glass*-responsive promoters/enhancers (Quiring *et al.*, Science (1994) 265:785-789). Wing tissue specific enhancers/promoters can be derived from the *dpp* or *vestigal* genes (Staehling-Hampton *et al.*, Cell Growth Differ. (1994) 5(6):585-593; Kim *et al.*, Nature (1996) 382:133-138). Finally, where it is necessary to restrict the activity of dominant active or dominant negative transgenes to regions where p53 is normally active, it
20 may be useful to use endogenous p53 promoters. The ectopic expression of DMp53 in *Drosophila* larval eye using *glass*-responsive enhancer elements is described in Example 12 below.

In *Drosophila*, binary control systems that employ exogenous DNA are useful when testing the mis-expression of genes in a wide variety of developmental stage-specific and
25 tissue-specific patterns. Two examples of binary exogenous regulatory systems include the UAS/GAL4 system from yeast (Hay *et al.*, PNAS (1997) 94(10):5195-5200; Ellis *et al.*, Development (1993) 119(3):855-865), and the "Tet system" derived from *E. coli* (Bello *et al.*, Development (1998) 125:2193-2202). The UAS/GAL4 system is a well-established and powerful method of mis-expression which employs the UAS_G upstream regulatory
30 sequence for control of promoters by the yeast GAL4 transcriptional activator protein (Brand and Perrimon, Development (1993) 118(2):401-15). In this approach, transgenic *Drosophila*, termed "target" lines, are generated where the gene of interest to be mis-expressed is operably fused to an appropriate promoter controlled by UAS_G. Other transgenic *Drosophila* strains, termed "driver" lines, are generated where the GAL4 coding

region is operably fused to promoters/enhancers that direct the expression of the GAL4 activator protein in specific tissues, such as the eye, wing, nervous system, gut, or musculature. The gene of interest is not expressed in the target lines for lack of a transcriptional activator to drive transcription from the promoter joined to the gene of interest. However, when the UAS-target line is crossed with a GAL4 driver line, mis-expression of the gene of interest is induced in resulting progeny in a specific pattern that is characteristic for that GAL4 line. The technical simplicity of this approach makes it possible to sample the effects of directed mis-expression of the gene of interest in a wide variety of tissues by generating one transgenic target line with the gene of interest, and crossing that target line with a panel of pre-existing driver lines.

In the "Tet" binary control system, transgenic *Drosophila* driver lines are generated where the coding region for a tetracycline-controlled transcriptional activator (tTA) is operably fused to promoters/enhancers that direct the expression of tTA in a tissue-specific and/or developmental stage-specific manner. The driver lines are crossed with transgenic *Drosophila* target lines where the coding region for the gene of interest to be mis-expressed is operably fused to a promoter that possesses a tTA-responsive regulatory element. When the resulting progeny are supplied with food supplemented with a sufficient amount of tetracycline, expression of the gene of interest is blocked. Expression of the gene of interest can be induced at will simply by removal of tetracycline from the food. Also, the level of expression of the gene of interest can be adjusted by varying the level of tetracycline in the food. Thus, the use of the Tet system as a binary control mechanism for mis-expression has the advantage of providing a means to control the amplitude and timing of mis-expression of the gene of interest, in addition to spatial control. Consequently, if a p53 gene has lethal or deleterious effects when mis-expressed at an early stage in development, such as the embryonic or larval stages, the function of the gene in the adult can still be assessed by adding tetracycline to the food during early stages of development and removing tetracycline later so as to induce mis-expression only at the adult stage.

Dominant negative mutations, by which the mutation causes a protein to interfere with the normal function of a wild-type copy of the protein, and which can result in loss-of-function or reduced-function phenotypes in the presence of a normal copy of the gene, can be made using known methods (Hershkowitz, *Nature* (1987) 329:219-222). In the case of active monomeric proteins, overexpression of an inactive form, achieved, for example, by linking the mutant gene to a highly active promoter, can cause competition for natural substrates or ligands sufficient to significantly reduce net activity of the normal protein.

Alternatively, changes to active site residues can be made to create a virtually irreversible association with a target.

Assays for Change in Gene Expression

5 Various expression analysis techniques may be used to identify genes which are differentially expressed between a cell line or an animal expressing a wild type p53 gene compared to another cell line or animal expressing a mutant p53 gene. Such expression profiling techniques include differential display, serial analysis of gene expression (SAGE), transcript profiling coupled to a gene database query, nucleic acid array technology,
10 subtractive hybridization, and proteome analysis (*e.g.* mass-spectrometry and two-dimensional protein gels). Nucleic acid array technology may be used to determine the genome-wide expression pattern in a normal animal for comparison with an animal having a mutation in the p53 gene. Gene expression profiling can also be used to identify other genes or proteins that may have a functional relation to p53. The genes are identified by
15 detecting changes in their expression levels following mutation, over-expression, under-expression, mis-expression or knock-out, of the p53 gene.

Phenotypes Associated With p53 Gene Mutations

 After isolation of model animals carrying mutated or mis-expressed p53 genes or
20 inhibitory RNAs, animals are carefully examined for phenotypes of interest. For analysis of p53 genes that have been mutated, animal models that are both homozygous and heterozygous for the altered p53 gene are analyzed. Examples of specific phenotypes that may be investigated include lethality; sterility; feeding behavior, tumor formation, perturbations in neuromuscular function including alterations in motility, and alterations in
25 sensitivity to pharmaceuticals. Some phenotypes more specific to flies include alterations in: adult behavior such as, flight ability, walking, grooming, phototaxis, mating or egg-laying; alterations in the responses of sensory organs, changes in the morphology, size or number of adult tissues such as, eyes, wings, legs, bristles, antennae, gut, fat body, gonads, and musculature; larval tissues such as mouth parts, cuticles, internal tissues or imaginal
30 discs; or larval behavior such as feeding, molting, crawling, or puparian formation; or developmental defects in any germline or embryonic tissues.

 Genomic sequences containing a p53 gene can be used to engineer an existing mutant insect line, using the transgenesis methods previously described, to determine whether the mutation is in the p53 gene. Briefly, germline transformants are crossed for

complementation testing to an existing or newly created panel of insect lines whose mutations have been mapped to the vicinity of the gene of interest (Fly Pushing, *supra*). If a mutant line is discovered to be rescued by the genomic fragment, as judged by complementation of the mutant phenotype, then the mutant line likely harbors a mutation in the p53 gene. This prediction can be further confirmed by sequencing the p53 gene from the mutant line to identify the lesion in the p53 gene.

Identification of Genes That Modify p53 Genes

The characterization of new phenotypes created by mutations or misexpression in p53 genes enables one to test for genetic interactions between p53 genes and other genes that may participate in the same, related, or interacting genetic or biochemical pathway(s). Individual genes can be used as starting points in large-scale genetic modifier screens as described in more detail below. Alternatively, RNAi methods can be used to simulate loss-of-function mutations in the genes being analyzed. It is of particular interest to investigate whether there are any interactions of p53 genes with other well-characterized genes, particularly genes involved in regulation of the cell cycle or apoptosis.

Genetic Modifier Screens

A genetic modifier screen using invertebrate model organisms is a particularly preferred method for identifying genes that interact with p53 genes, because large numbers of animals can be systematically screened making it more possible that interacting genes will be identified. In *Drosophila*, a screen of up to about 10,000 animals is considered to be a pilot-scale screen. Moderate-scale screens usually employ about 10,000 to about 50,000 flies, and large-scale screens employ greater than about 50,000 flies. In a genetic modifier screen, animals having a mutant phenotype due to a mutation in or misexpression of the p53 gene are further mutagenized, for example by chemical mutagenesis or transposon mutagenesis.

The procedures involved in typical *Drosophila* genetic modifier screens are well-known in the art (Wolfner and Goldberg, *Methods in Cell Biology* (1994) 44:33-80; and Karim *et al.*, *Genetics* (1996) 143:315-329). The procedures used differ depending upon the precise nature of the mutant allele being modified. If the mutant allele is genetically recessive, as is commonly the situation for a loss-of-function allele, then most typically males, or in some cases females, which carry one copy of the mutant allele are exposed to an effective mutagen, such as EMS, MMS, ENU, triethylamine, diepoxylkanes, ICR-170, formaldehyde, X-rays, gamma rays, or ultraviolet radiation. The mutagenized animals are

crossed to animals of the opposite sex that also carry the mutant allele to be modified. In the case where the mutant allele being modified is genetically dominant, as is commonly the situation for ectopically expressed genes, wild type males are mutagenized and crossed to females carrying the mutant allele to be modified.

5 The progeny of the mutagenized and crossed flies that exhibit either enhancement or suppression of the original phenotype are presumed to have mutations in other genes, called "modifier genes", that participate in the same phenotype-generating pathway. These progeny are immediately crossed to adults containing balancer chromosomes and used as founders of a stable genetic line. In addition, progeny of the founder adult are retested
10 under the original screening conditions to ensure stability and reproducibility of the phenotype. Additional secondary screens may be employed, as appropriate, to confirm the suitability of each new modifier mutant line for further analysis.

Standard techniques used for the mapping of modifiers that come from a genetic screen in *Drosophila* include meiotic mapping with visible or molecular genetic markers;
15 male-specific recombination mapping relative to P-element insertions; complementation analysis with deficiencies, duplications, and lethal P-element insertions; and cytological analysis of chromosomal aberrations (Fly Pushing, *supra*). Genes corresponding to modifier mutations that fail to complement a lethal P-element may be cloned by plasmid rescue of the genomic sequence surrounding that P-element. Alternatively, modifier genes
20 may be mapped by phenotype rescue and positional cloning (Sambrook *et al.*, *supra*).

Newly identified modifier mutations can be tested directly for interaction with other genes of interest known to be involved or implicated with p53 genes using methods described above. Also, the new modifier mutations can be tested for interactions with genes in other pathways that are not believed to be related to regulation of cell cycle or apoptosis.
25 New modifier mutations that exhibit specific genetic interactions with other genes implicated in cell cycle regulation or apoptosis, and not with genes in unrelated pathways, are of particular interest.

The modifier mutations may also be used to identify "complementation groups". Two modifier mutations are considered to fall within the same complementation group if
30 animals carrying both mutations in trans exhibit essentially the same phenotype as animals that are homozygous for each mutation individually and, generally are lethal when in trans to each other (Fly Pushing, *supra*). Generally, individual complementation groups defined in this way correspond to individual genes.

When p53 modifier genes are identified, homologous genes in other species can be isolated using procedures based on cross-hybridization with modifier gene DNA probes, PCR-based strategies with primer sequences derived from the modifier genes, and/or computer searches of sequence databases. For therapeutic applications related to the function of p53 genes, human and rodent homologs of the modifier genes are of particular interest.

Although the above-described *Drosophila* genetic modifier screens are quite powerful and sensitive, some genes that interact with p53 genes may be missed in this approach, particularly if there is functional redundancy of those genes. This is because the vast majority of the mutations generated in the standard mutagenesis methods will be loss-of-function mutations, whereas gain-of-function mutations that could reveal genes with functional redundancy will be relatively rare. Another method of genetic screening in *Drosophila* has been developed that focuses specifically on systematic gain-of-function genetic screens (Rorth *et al.*, Development (1998) 125:1049-1057). This method is based on a modular mis-expression system utilizing components of the GAL4/UAS system (described above) where a modified P element, termed an "enhanced P" (EP) element, is genetically engineered to contain a GAL4-responsive UAS element and promoter. Any other transposons can also be used for this system. The resulting transposon is used to randomly tag genes by insertional mutagenesis (similar to the method of P element mutagenesis described above). Thousands of transgenic *Drosophila* strains, termed EP lines, can be generated, each containing a specific UAS-tagged gene. This approach takes advantage of the preference of P elements to insert at the 5'-ends of genes. Consequently, many of the genes that are tagged by insertion of EP elements become operably fused to a GAL4-regulated promoter, and increased expression or mis-expression of the randomly tagged gene can be induced by crossing in a GAL4 driver gene.

Systematic gain-of-function genetic screens for modifiers of phenotypes induced by mutation or mis-expression of a p53 gene can be performed by crossing several thousand *Drosophila* EP lines individually into a genetic background containing a mutant or mis-expressed p53 gene, and further containing an appropriate GAL4 driver transgene. It is also possible to remobilize the EP elements to obtain novel insertions. The progeny of these crosses are then analyzed for enhancement or suppression of the original mutant phenotype as described above. Those identified as having mutations that interact with the p53 gene can be tested further to verify the reproducibility and specificity of this genetic interaction. EP insertions that demonstrate a specific genetic interaction with a mutant or mis-expressed

p53 gene, have a physically tagged new gene which can be identified and sequenced using PCR or hybridization screening methods, allowing the isolation of the genomic DNA adjacent to the position of the EP element insertion.

5 **Identification of Molecules that Interact With p53**

A variety of methods can be used to identify or screen for molecules, such as proteins or other molecules, that interact with p53 protein, or derivatives or fragments thereof. The assays may employ purified p53 protein, or cell lines or a model organism such as *Drosophila* that has been genetically engineered to express p53 protein. Suitable
10 screening methodologies are well known in the art to test for proteins and other molecules that interact with a gene/protein of interest (see *e.g.*, PCT International Publication No. WO 96/34099). The newly identified interacting molecules may provide new targets for pharmaceutical agents. Any of a variety of exogenous molecules, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides, or phage display libraries),
15 may be screened for binding capacity. In a typical binding experiment, the p53 protein or fragment is mixed with candidate molecules under conditions conducive to binding, sufficient time is allowed for any binding to occur, and assays are performed to test for bound complexes. A variety of assays to find interacting proteins are known in the art, for example, immunoprecipitation with an antibody that binds to the protein in a complex
20 followed by analysis by size fractionation of the immunoprecipitated proteins (*e.g.* by denaturing or nondenaturing polyacrylamide gel electrophoresis), Western analysis, non-denaturing gel electrophoresis, *etc.*

Two-hybrid assay systems

A preferred method for identifying interacting proteins is a two-hybrid assay system
25 or variation thereof (Fields and Song, *Nature* (1989) 340:245-246; U.S. Pat. No. 5,283,173; for review see Brent and Finley, *Annu. Rev. Genet.* (1997) 31:663-704). The most commonly used two-hybrid screen system is performed using yeast. All systems share three elements: 1) a gene that directs the synthesis of a "bait" protein fused to a DNA binding domain; 2) one or more "reporter" genes having an upstream binding site for the
30 bait, and 3) a gene that directs the synthesis of a "prey" protein fused to an activation domain that activates transcription of the reporter gene. For the screening of proteins that interact with p53 protein, the "bait" is preferably a p53 protein, expressed as a fusion protein to a DNA binding domain; and the "prey" protein is a protein to be tested for ability to interact with the bait, and is expressed as a fusion protein to a transcription activation

domain. The prey proteins can be obtained from recombinant biological libraries expressing random peptides.

The bait fusion protein can be constructed using any suitable DNA binding domain, such as the *E. coli* LexA repressor protein, or the yeast GAL4 protein (Bartel *et al.*, BioTechniques (1993) 14:920-924; Chasman *et al.*, Mol. Cell. Biol. (1989) 9:4746-4749; 5 Ma *et al.*, Cell (1987) 48:847-853; Ptashne *et al.*, Nature (1990) 346:329-331). The prey fusion protein can be constructed using any suitable activation domain such as GAL4, VP-16, *etc.* The preys may contain useful moieties such as nuclear localization signals (Ylikomi *et al.*, EMBO J. (1992) 11:3681-3694; Dingwall and Laskey, Trends Biochem. 10 Sci. Trends Biochem. Sci. (1991) 16:479-481) or epitope tags (Allen *et al.*, Trends Biochem. Sci. Trends Biochem. Sci. (1995) 20:511-516) to facilitate isolation of the encoded proteins. Any reporter gene can be used that has a detectable phenotype such as reporter genes that allow cells expressing them to be selected by growth on appropriate medium (*e.g.* HIS3, LEU2 described by Chien *et al.*, PNAS (1991) 88:9572-9582; and 15 Gyuris *et al.*, Cell (1993) 75:791-803). Other reporter genes, such as LacZ and GFP, allow cells expressing them to be visually screened (Chien *et al.*, *supra*).

Although the preferred host for two-hybrid screening is the yeast, the host cell in which the interaction assay and transcription of the reporter gene occurs can be any cell, such as mammalian (*e.g.* monkey, mouse, rat, human, bovine), chicken, bacterial, or insect 20 cells. Various vectors and host strains for expression of the two fusion protein populations in yeast can be used (U.S. Pat. No. 5,468,614; Bartel *et al.*, Cellular Interactions in Development (1993) Hartley, ed., Practical Approach Series xviii, IRL Press at Oxford University Press, New York, NY, pp. 153-179; and Fields and Sternglanz, Trends In Genetics (1994) 10:286-292). As an example of a mammalian system, interaction of 25 activation tagged VP16 derivatives with a GAL4-derived bait drives expression of reporters that direct the synthesis of hygromycin B phosphotransferase, chloramphenicol acetyltransferase, or CD4 cell surface antigen (Fearon *et al.*, PNAS (1992) 89:7958-7962). As another example, interaction of VP16-tagged derivatives with GAL4-derived baits drives the synthesis of SV40 T antigen, which in turn promotes the replication of the prey 30 plasmid, which carries an SV40 origin (Vasavada *et al.*, PNAS (1991) 88:10686-10690).

Typically, the bait p53 gene and the prey library of chimeric genes are combined by mating the two yeast strains on solid or liquid media for a period of approximately 6-8 hours. The resulting diploids contain both kinds of chimeric genes, i.e., the DNA-binding domain fusion and the activation domain fusion. Transcription of the reporter gene can be

detected by a linked replication assay in the case of SV40 T antigen (Vasavada *et al.*, *supra*) or using immunoassay methods (Alam and Cook. Anal. Biochem. (1990)188:245-254).

The activation of other reporter genes like URA3, HIS3, LYS2, or LEU2 enables the cells to grow in the absence of uracil, histidine, lysine, or leucine, respectively, and hence serves
5 as a selectable marker. Other types of reporters are monitored by measuring a detectable signal. For example, GFP and lacZ have gene products that are fluorescent and chromogenic, respectively.

After interacting proteins have been identified, the DNA sequences encoding the proteins can be isolated. In one method, the activation domain sequences or DNA-binding
10 domain sequences (depending on the prey hybrid used) are amplified, for example, by PCR using pairs of oligonucleotide primers specific for the coding region of the DNA binding domain or activation domain. If a shuttle (yeast to *E. coli*) vector is used to express the fusion proteins, the DNA sequences encoding the proteins can be isolated by transformation of *E. coli* using the yeast DNA and recovering the plasmids from *E. coli*. Alternatively, the
15 yeast vector can be isolated, and the insert encoding the fusion protein subcloned into a bacterial expression vector, for growth of the plasmid in *E. coli*.

Antibodies and Immunoassay

p53 proteins encoded by any of SEQ ID NOs:2, 4, 6, 8, or 10 and derivatives and fragments thereof, such as those discussed above, may be used as an immunogen to
20 generate monoclonal or polyclonal antibodies and antibody fragments or derivatives (*e.g.* chimeric, single chain, Fab fragments). For example, fragments of a p53 protein, preferably those identified as hydrophilic, are used as immunogens for antibody production using art-known methods such as by hybridomas; production of monoclonal antibodies in germ-free animals (PCT/US90/02545); the use of human hybridomas (Cole *et al.*, PNAS (1983)
25 80:2026-2030; Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy (1985) Alan R. Liss, pp. 77-96), and production of humanized antibodies (Jones *et al.*, Nature (1986) 321:522-525; U.S. Pat. 5,530,101). In a particular embodiment, p53 polypeptide fragments provide specific antigens and/or immunogens, especially when coupled to carrier proteins. For example, peptides are covalently coupled to keyhole limpet antigen (KLH) and the
30 conjugate is emulsified in Freund's complete adjuvant. Laboratory rabbits are immunized according to conventional protocol and bled. The presence of specific antibodies is assayed by solid phase immunosorbent assays using immobilized corresponding polypeptide. Specific activity or function of the antibodies produced may be determined by convenient *in vitro*, cell-based, or *in vivo* assays: *e.g.* *in vitro* binding assays, *etc.* Binding affinity may be

assayed by determination of equilibrium constants of antigen-antibody association (usually at least about 10^7 M^{-1} , preferably at least about 10^8 M^{-1} , more preferably at least about 10^9 M^{-1}). Example 11 below further describes the generation of anti-DMp53 antibodies.

Immunoassays can be used to identify proteins that interact with or bind to p53 protein. Various assays are available for testing the ability of a protein to bind to or compete with binding to a wild-type p53 protein or for binding to an anti-p53 protein antibody. Suitable assays include radioimmunoassays, ELISA (enzyme linked immunosorbent assay), immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, *etc.*

Identification of Potential Drug Targets

Once new p53 genes or p53 interacting genes are identified, they can be assessed as potential drug or pesticide targets using animal models such as *Drosophila* or other insects, or using cells that express endogenous p53, or that have been engineered to express p53.

Assays of Compounds on Insects

Potential insecticidal compounds can be administered to insects in a variety of ways, including orally (including addition to synthetic diet, application to plants or prey to be consumed by the test organism), topically (including spraying, direct application of compound to animal, allowing animal to contact a treated surface), or by injection. Insecticides are typically very hydrophobic molecules and must commonly be dissolved in organic solvents, which are allowed to evaporate in the case of methanol or acetone, or at low concentrations can be included to facilitate uptake (ethanol, dimethyl sulfoxide).

The first step in an insect assay is usually the determination of the minimal lethal dose (MLD) on the insects after a chronic exposure to the compounds. The compounds are usually diluted in DMSO, and applied to the food surface bearing 0-48 hour old embryos and larvae. In addition to MLD, this step allows the determination of the fraction of eggs that hatch, behavior of the larvae, such as how they move /feed compared to untreated larvae, the fraction that survive to pupate, and the fraction that eclose (emergence of the adult insect from puparium). Based on these results more detailed assays with shorter exposure times may be designed, and larvae might be dissected to look for obvious

morphological defects. Once the MLD is determined, more specific acute and chronic assays can be designed.

In a typical acute assay, compounds are applied to the food surface for embryos, larvae, or adults, and the animals are observed after 2 hours and after an overnight incubation. For application on embryos, defects in development and the percent that survive to adulthood are determined. For larvae, defects in behavior, locomotion, and molting may be observed. For application on adults, behavior and neurological defects are observed, and effects on fertility are noted. Any deleterious effect on insect survival, motility and fertility indicates that the compound has utility in controlling pests.

For a chronic exposure assay, adults are placed on vials containing the compounds for 48 hours, then transferred to a clean container and observed for fertility, neurological defects, and death.

Assay of Compounds using Cell Cultures

Compounds that modulate (*e.g.* block or enhance) p53 activity may be tested on cells expressing endogenous normal or mutant p53s, and/or on cells transfected with vectors that express p53, or derivatives or fragments of p53. The compounds are added at varying concentration and their ability to modulate the activity of p53 genes is determined using any of the assays for tumor suppressor genes described above (*e.g.* by measuring transcription activity, apoptosis, proliferation/cell cycle, and/or transformation). Compounds that selectively modulate p53 are identified as potential drug candidates having p53 specificity.

Identification of small molecules and compounds as potential pharmaceutical compounds from large chemical libraries requires high-throughput screening (HTS) methods (Bolger, *Drug Discovery Today* (1999) 4:251-253). Several of the assays mentioned herein can lend themselves to such screening methods. For example, cells or cell lines expressing wild type or mutant p53 protein or its fragments, and a reporter gene can be subjected to compounds of interest, and depending on the reporter genes, interactions can be measured using a variety of methods such as color detection, fluorescence detection (*e.g.* GFP), autoradiography, scintillation analysis, *etc.*

Agricultural uses of insect p53 sequences

Insect p53 genes may be used in controlling agriculturally important pest species. For example, the proteins, genes, and RNAs disclosed herein, or their fragments may have activity in modifying the growth, feeding and/or reproduction of crop-damaging insects, or insect pests of farm animals or of other animals. In general, effective pesticides exert a

disabling activity on the target pest such as lethality, sterility, paralysis, blocked development, or cessation of feeding. Such pests include egg, larval, juvenile and adult forms of flies, mosquitos, fleas, moths, beetles, cicadia, grasshoppers, aphids and crickets. The functional analyses of insect p53 genes described herein has revealed roles for these genes and proteins in controlling apoptosis, response to DNA damaging agents, and protection of cells of the germline. Since overexpression of DMp53 induces apoptosis in *Drosophila*, the insect p53 genes and proteins in an activated form have application as "cell death" genes which if delivered to or expressed in specific target tissues such as the gut, nervous system, or gonad, would have a use in controlling insect pests. Alternatively, since DMp53 plays a role in response to DNA damaging agents such as X-rays, interference with p53 function in insects has application in sensitizing insects to DNA damaging agents for sterilization. For example, current methods for controlling pest populations through the release of irradiated insects into the environment (Knippling, J Econ Ent (1955) 48: 459-462; Knippling (1979) U.S. Dept. Agric. Handbook No. 512) could be improved by causing expression of dominant negative forms of p53 genes, proteins, or RNAs in insects and most preferably germline tissue of insects, or by exposing insects to chemical compounds which block p53 function.

Mutational analysis of insect p53 proteins may also be used in connection with the control of agriculturally-important pests. In this regard, mutational analysis of insect p53 genes provides a rational approach to determine the precise biological function of this class of proteins in invertebrates. Further, mutational analysis coupled with large-scale systematic genetic modifier screens provides a means to identify and validate other potential pesticide targets that might be constituents of the p53 signaling pathway. Tests for pesticidal activities can be any method known in the art. Pesticides comprising the nucleic acids of the insect p53 proteins may be prepared in a suitable vector for delivery to a plant or animal. Such vectors include *Agrobacterium tumefaciens* Ti plasmid-based vectors for the generation of transgenic plants (Horsch *et al.*, Proc Natl Acad Sci U S A. (1986) 83(8):2571-2575; Fraley *et al.*, Proc. Natl. Acad. Sci. USA (1983) 80:4803) or recombinant cauliflower mosaic virus for the inoculation of plant cells or plants (U.S. Pat No. 4,407,956); retrovirus based vectors for the introduction of genes into vertebrate animals (Burns *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90:8033-37); and vectors based on transposable elements for incorporation into invertebrate animals using vectors and methods already described above. For example, transgenic insects can be generated using a transgene comprising a p53 gene operably fused to an appropriate inducible promoter, such

as a tTA-responsive promoter, in order to direct expression of the tumor suppressor protein at an appropriate time in the life cycle of the insect. In this way, one may test efficacy as an insecticide in, for example, the larval phase of the life cycle (*e.g.*, when feeding does the greatest damage to crops).

5 Recombinant or synthetic p53 proteins, RNAs or their fragments, in wild-type or mutant forms, can be assayed for insecticidal activity by injection of solutions of p53 proteins or RNAs into the hemolymph of insect larvae (Blackburn, *et al.*, Appl. Environ. Microbiol. (1998) 64(8):3036-41; Bowen and Ensign, Appl. Environ. Microbiol. (1998) 64(8):3029-35). Further, transgenic plants that express p53 proteins or RNAs or their
10 fragments can be tested for activity against insect pests (Estruch *et al.*, Nat. Biotechnol. (1997) 15(2):137-41).

 Insect p53 genes may be used as insect control agents in the form of recombinant viruses that direct the expression of a tumor suppressor gene in the target pest. A variety of suitable recombinant virus systems for expression of proteins in infected insect cells are
15 well known in the art. A preferred system uses recombinant baculoviruses. The use of recombinant baculoviruses as a means to engineer expression of toxic proteins in insects, and as insect control agents, has a number of specific advantages including host specificity, environmental safety, the availability of vector systems, and the potential use of the recombinant virus directly as a pesticide without the need for purification or formulation of
20 the tumor suppressor protein (Cory and Bishop, Mol. Biotechnol. (1997) 7(3):303-13; and U.S. Pat. Nos. 5,470,735; 5,352,451; 5,770,192; 5,759,809; 5,665,349; and 5,554,592). Thus, recombinant baculoviruses that direct the expression of insect p53 genes can be used for both testing the pesticidal activity of tumor suppressor proteins under controlled laboratory conditions, and as insect control agents in the field. One disadvantage of wild
25 type baculoviruses as insect control agents can be the amount of time between application of the virus and death of the target insect, typically one to two weeks. During this period, the insect larvae continue to feed and damage crops. Consequently, there is a need to develop improved baculovirus-derived insect control agents which result in a rapid cessation of feeding of infected target insects. The cell cycle and apoptotic regulatory roles
30 of p53 in vertebrates raises the possibility that expression of tumor suppressor proteins from recombinant baculovirus in infected insects may have a desirable effect in controlling metabolism and limiting feeding of insect pests.

 Insect p53 genes, RNAs, proteins or fragments may be formulated with any carrier suitable for agricultural use, such as water, organic solvents and/or inorganic solvents. The

pesticide composition may be in the form of a solid or liquid composition and may be prepared by fundamental formulation processes such as dissolving, mixing, milling, granulating, and dispersing. Compositions may contain an insect p53 protein or gene in a mixture with agriculturally acceptable excipients such as vehicles, carriers, binders, UV blockers, adhesives, hemecants, thickeners, dispersing agents, preservatives and insect attractants. Thus the compositions of the invention may, for example, be formulated as a solid comprising the active agent and a finely divided solid carrier. Alternatively, the active agent may be contained in liquid compositions including dispersions, emulsions and suspensions thereof. Any suitable final formulation may be used, including for example, granules, powder, bait pellets (a solid composition containing the active agent and an insect attractant or food substance), microcapsules, water dispersible granules, emulsions and emulsified concentrates. Examples of adjuvant or carriers suitable for use with the present invention include water, organic solvent, inorganic solvent, talc, pyrophyllite, synthetic fine silica, attapugus clay, kieselguhr chalk, diatomaceous earth, lime, calcium carbonate, bontonite, fuller's earth, cottonseed hulls, wheat flour, soybean flour, pumice, tripoli, wood flour, walnut shell flour, redwood flour, and lignin. The compositions may also include conventional insecticidal agents and/or may be applied in conjunction with conventional insecticidal agents.

20 EXAMPLES

The following examples describe the isolation and cloning of the nucleic acid sequence of SEQ ID NOs: 1, 3, 5, 7, 9, and 18, and how these sequences, derivatives and fragments thereof, and gene products can be used for genetic studies to elucidate mechanisms of the p53 pathway as well as the discovery of potential pharmaceutical agents that interact with the pathway.

These Examples are provided merely as illustrative of various aspects of the invention and should not be construed to limit the invention in any way.

Example 1: Preparation of *Drosophila* cDNA Library

30 A *Drosophila* expressed sequence tag (EST) cDNA library was prepared as follows. Tissue from mixed stage embryos (0-20 hour), imaginal disks and adult fly heads were collected and total RNA was prepared. Mitochondrial rRNA was removed from the total RNA by hybridization with biotinylated rRNA specific oligonucleotides and the resulting RNA was selected for polyadenylated mRNA. The resulting material was then used to

construct a random primed library. First strand cDNA synthesis was primed using a six nucleotide random primer. The first strand cDNA was then tailed with terminal transferase to add approximately 15 dGTP molecules. The second strand was primed using a primer which contained a NotI site followed by a 13 nucleotide C-tail to hybridize to the G-tailed
5 first strand cDNA. The double stranded cDNA was ligated with BstXI adaptors and digested with NotI. The cDNA was then fractionated by size by electrophoresis on an agarose gel and the cDNA greater than 700 bp was purified. The cDNA was ligated with NotI, BstXI digested pCDNA-sk+ vector (a derivative of pBluescript, Stratagene) and used to transform *E. coli* (XL1blue). The final complexity of the library was 6×10^6
10 independent clones.

The cDNA library was normalized using a modification of the method described by Bonaldo *et al.* (Genome Research (1996) 6:791-806). Biotinylated driver was prepared from the cDNA by PCR amplification of the inserts and allowed to hybridize with single stranded plasmids of the same library. The resulting double-stranded forms were removed
15 using streptavidin magnetic beads, the remaining single stranded plasmids were converted to double stranded molecules using Sequenase (Amersham, Arlington Hills, IL), and the plasmid DNA stored at -20°C prior to transformation. Aliquots of the normalized plasmid library were used to transform *E. coli* (XL1blue or DH10B), plated at moderate density, and the colonies picked into a 384-well master plate containing bacterial growth media using a
20 Qbot robot (Genetix, Christchurch, UK). The clones were allowed to grow for 24 hours at 37° C then the master plates were frozen at -80° C for storage. The total number of colonies picked for sequencing from the normalized library was 240,000. The master plates were used to inoculate media for growth and preparation of DNA for use as template in sequencing reactions. The reactions were primarily carried out with primer that initiated at
25 the 5' end of the cDNA inserts. However, a minor percentage of the clones were also sequenced from the 3' end. Clones were selected for 3' end sequencing based on either further biological interest or the selection of clones that could extend assemblies of contiguous sequences ("contigs") as discussed below. DNA sequencing was carried out using ABI377 automated sequencers and used either ABI FS, dirhodamine or BigDye
30 chemistries (Applied Biosystems, Inc., Foster City, CA).

Analysis of sequences was done as follows: the traces generated by the automated sequencers were base-called using the program "Phred" (Gordon, Genome Res. (1998) 8:195-202), which also assigned quality values to each base. The resulting sequences were

trimmed for quality in view of the assigned scores. Vector sequences were also removed. Each sequence was compared to all other fly EST sequences using the BLAST program and a filter to identify regions of near 100% identity. Sequences with potential overlap were then assembled into contigs using the programs "Phrap", "Phred" and "Consed" (Phil Green, University of Washington, Seattle, Washington; <http://bozeman.mbt.washington.edu/phrap.docs/phrap.html>). The resulting assemblies were then compared to existing public databases and homology to known proteins was then used to direct translation of the consensus sequence. Where no BLAST homology was available, the statistically most likely translation based on codon and hexanucleotide preference was used. The Pfam (Bateman *et al.*, Nucleic Acids Res. (1999) 27:260-262) and Prosite (Hofmann *et al.*, Nucleic Acids Res. (1999) 27(1):215-219) collections of protein domains were used to identify motifs in the resulting translations. The contig sequences were archived in an Oracle-based relational database (FlyTag™, Exelixis Pharmaceuticals, Inc., South San Francisco, CA).

15

Example 2: Other cDNA libraries

A *Leptinotarsa* (Colorado Potato Beetle) library was prepared using the Lambda ZAP cDNA cloning kit from Stratagene (Stratagene, La Jolla, CA, cat#200450), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt primed using mRNA from mixed stage larvae *Leptinotarsa*.

A *Tribolium* library was made using pSPORT cDNA library construction system (Life Technologies, Gaithersburg, MD), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt primed using mRNA from adult *Tribolium*.

Example 3: Cloning of the p53 nucleic acid from *Drosophila* (DMp53)

The TBLASTN program (Altschul *et al.*, *supra*) was used to query the FlyTag™ database with a squid p53 protein sequence (GenBank gi:1244762), chosen because the squid sequence was one of only two members of the p53 family that had been identified previously from an invertebrate. The results revealed a single sequence contig, which was 960 bp in length and which exhibited highly significant homology to squid p53 (score=192, $P=5.1 \times 10^{-12}$). Further analysis of this sequence with the BLASTX program against GenBank protein sequences demonstrated that this contig exhibited significant homology to the entire known family of p53-like sequences in vertebrates, and that it contained coding

sequences homologous to the p53 family that encompassed essentially all of the DNA-binding domain, which is the most conserved region of the p53 protein family. Inspection of this contig indicated that it was an incomplete cDNA, missing coding regions C-terminal to the presumptive DNA-binding domain as well as the 3' untranslated region of the mRNA.

5 The full-length cDNA clone was produced by Rapid Amplification of cDNA ends (RACE: Frohman *et al.*, PNAS (1988) 85:8998-9002). A RACE-ready library was generated from Clontech (Palo Alto, CA) *Drosophila* embryo poly A⁺ RNA (Cat#694-1) using Clontech's Marathon cDNA amplification kit (Cat# K1802), and following manufacturer's directions. The following primers were used on the library to retrieve full-
10 length clones:

3'373	CCATGCTGAAGCAATAACCAACCGATG	SEQ ID NO:11
3'510	GGAACACACGCAAATTAAGTGGTTGGATGG	SEQ ID NO:12
3'566	TGATTTTGACAGCGGACCAACGGG	SEQ ID NO:13
15 3'799	GGAAGTTTCTTTTCGCCCCGATACACGAG	SEQ ID NO:14
5'164	GGCACAAAGAAAGCACTGATTCCGAGG	SEQ ID NO:15
5'300	GGAATCTGATGCAGTTCAGCCAGCAATC	SEQ ID NO:16
5'932	GGATCGCATCCAAGACGAACGCC	SEQ ID NO:17

20 RACE reactions to obtain additional 5' and 3' sequence of the *Drosophila* p53 cDNA were performed as follows. Each RACE reaction contained: 40 µl of H₂O, 5 µl of 10X Advantage PCR buffer (Clontech), 1 µl of specific p53 RACE primer at 10 µM, 1 µl of AP1 primer (from Clontech Marathon kit) at 10 µM, 1 µl of cDNA, 1 µl of dNTPs at 5 mM, 1 µl of Advantage DNA polymerase (Clontech). For 5' RACE, the reactions
25 contained either the 3'373, 3'510, 3'566, or 3'799 primers. For 3' RACE, the reactions contained either the 5'164 or 5'300 primers. The reaction mixtures were subjected to the following thermocycling program steps for touchdown PCR: (1) 94°C 1 min, (2) 94°C 0.5 min, (3) 72°C 4 min, (4) repeat steps 2-3 four times, (5) 94°C 0.5 min, (6) 70°C 4 min, (7) repeat steps 5-6 four times, (8) 94°C 0.33 min, (9) 68°C 4 min, (10) repeat steps 8-9 24
30 times, (11) 68°C 4 min, (12) remain at 4°C.

Products of the RACE reactions were analyzed by gel electrophoresis. Discrete DNA species of the following sizes were observed in the RACE products produced with each of the following primers: 3'373, approx. 400 bp; 3'510, approx. 550 bp, 3'566, approx. 600 bp; 3'799, approx. 850 bp; 5'164, approx. 1400 bp, 5'300 approx. 1300 bp. The RACE

DNA products were cloned directly into the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, California) following the manufacturers directions. Colonies of transformed *E. coli* were picked for each construct, and plasmid DNA prepared using a QIAGEN tip 20 kit (QIAGEN, Valencia, California). Sequences of the RACE cDNA
5 inserts in within each clone were determined using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc. Foster City, California) and either M13 reverse or BigT7 primers for priming from flanking vector sequences, or 5932 or 3373 primers (described above) for priming internally from *Drosophila* p53 cDNA sequences. The products were analyzed using ABI 377 DNA sequencer. Sequences were assembled
10 into a contig using the Sequencher program (Gene Codes Corporation), and contained a single open reading frame encoding a predicted protein of 385 amino acids, which compared favorably with the known lengths of vertebrate p53 proteins, 363 to 396 amino acids (Soussi *et al.*, *Oncogene* (1990) 5:945-952). Analysis of the predicted *Drosophila* p53 protein using the BLASTP homology searching program and the GenBank database
15 confirmed that this protein was a member of the p53 family, since it exhibited highly significant homology to all known p53 related proteins, but no significant homology to other protein families.

Example 4: Cloning of p53 Nucleic Acid Sequences from other insects

20 The PCR conditions used for cloning the p53 nucleic acid sequences comprised a denaturation step of 94° C, 5 min; followed by 35 cycles of: 94° C 1 min, 55° C 1 min 72° C 1 min; then, a final extension at 72° C 10 min. All DNA sequencing reactions were performed using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc.) and products were analyzed using ABI 377 DNA sequencers. Trace data
25 obtained from the ABI 377 DNA sequencers was analyzed and assembled into contigs using the Phred-Phrap programs.

The DMp53 DNA and protein sequences were used to query sequences from *Tribolium*, *Leptinotarsa*, and *Heliothis* cDNA libraries using the BLAST computer program, and the results revealed several candidate cDNA clones that might encode p53
30 related sequences. For each candidate p53 cDNA clone, well-separated, single colonies were streaked on a plate and end-sequenced to verify the clones. Single colonies were picked and the plasmid DNA was purified using Qiagen REAL Preps (Qiagen, Inc., Valencia, CA). Samples were then digested with appropriate enzymes to excise insert from

vector and determine size. For example, the vector pOT2, (www.fruitfly.org/EST/pOT2vector.html) can be excised with XhoI/EcoRI; or pBluescript (Stratagene) can be excised with BssH II. Clones were then sequenced using a combination of primer walking and *in vitro* transposon tagging strategies.

5 For primer walking, primers were designed to the known DNA sequences in the clones, using the Primer-3 software (Steve Rozen. Helen J. Skaletsky (1998) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.) These primers were then used in sequencing reactions to extend the sequence until the full sequence of the insert was determined.

10 The GPS-1 Genome Priming System *in vitro* transposon kit (New England Biolabs, Inc., Beverly, MA) was used for transposon-based sequencing, following manufacturer's protocols. Briefly, multiple DNA templates with randomly interspersed primer-binding sites were generated. These clones were prepared by picking 24 colonies/clone into a Qiagen REAL Prep to purify DNA and sequenced by using supplied primers to perform
15 bidirectional sequencing from both ends of transposon insertion.

Sequences were then assembled using Phred/Phrap and analyzed using Consed. Ambiguities in the sequence were resolved by resequencing several clones. This effort resulted in several contiguous nucleotide sequences. For *Leptinotarsa*, a contig was assembled of 2601 bases in length, encompassing an open reading frame (ORF) of 1059
20 nucleotides encoding a predicted protein of 353 amino acids. The ORF extends from base 121-1180 of SEQ ID NO:3. For *Tribolium*, a contig was assembled of 1292 bases in length, encompassing an ORF of 1050 nucleotides, extending from base 95-1145 of SEQ ID NO:5, and encoding a predicted protein of 350 amino acids. The analysis of another candidate *Tribolium* p53 clone also generated a second contig of 509 bases in length, encompassing a
25 partial ORF of 509 nucleotides (SEQ ID NO: 7), and encoding a partial protein of 170 amino acids. For *Heliothis*, a contig was assembled of 434 bases in length, encompassing a partial ORF of 434 nucleotides (SEQ ID NO:9), and encoding a partial protein of 145 amino acids.

30 **Example 5: Northern Blot analysis of DMp53**

Northern blot analysis using standard methods was performed using three different poly(A)+ mRNA preparations. 0-12 h embryo. 12-24 h embryo. and adult, which were fractionated on an agarose gel along with size standards and blotted to a nylon membrane. A DNA fragment containing the entire *Drosophila* p53 coding region was excised by

HincII digestion, separated by electrophoresis in an agarose gel, extracted from the gel, and ³²P-labeled by random-priming using the Rediprime labeling system (Amersham, Piscataway, NJ). Hybridization of the labeled probe to the mRNA blot was performed overnight. The blot was washed at high stringency (0.2x SSC/0.1% SDS at 65°C) and mRNA species that specifically hybridized to the probe were detected by autoradiography using X-ray film. The results showed a single cross-hybridizing mRNA species of approximately 1.6 kilobases in all three mRNA sources. This data was consistent with the observed sizes of the 5' and 3' RACE products described above.

10 **Example 6: Cytogenetic mapping of the DMp53 gene**

It was of interest to identify the map location of the DMp53 gene in order to determine whether any existing *Drosophila* mutants correspond to mutations in the DMp53 gene, as well as for engineering new mutations within this gene. The cytogenetic location of the DMp53 gene was determined by *in situ* hybridization to polytene chromosomes (Pardue, Meth Cell Biol (1994) 44:333-351) following the protocol outlined below (steps A-C).

(A) Preparation of polytene chromosome squashes: Dissected salivary glands were placed into a drop of 45% acetic acid. Glands were transferred to drop of 1:2:3 mixture of lactic acid: water:acetic acid. Glands were then squashed between a cover slip and a slide and incubated at 4°C overnight. Squashes were frozen in liquid N₂ and the coverslip removed. Slides were then immediately immersed in 70% ethanol for 10 min. and then air dried. Slides were then heat treated for 30 min. at 68°C in 2x SSC buffer. Squashes were then dehydrated by treatment with 70% ethanol for 10 min. followed by 95% ethanol for 5 min.

25 (B) Preparation of a biotinylated hybridization probe: a solution was prepared by mixing: 50 µl of 1 M Tris-HCl pH 7.5, 6.35 µl of 1 M MgCl₂, 0.85 µl of beta-mercaptoethanol, 0.625 µl of 100 mM dATP, 0.625 µl of 100 mM dCTP, 0.625 µl of 100 mM dGTP, 125 µl of 2 M HEPES pH 6.6, and 75 µl of 10 mg/ml pd(N)₆ (Pharmacia, Kalamazoo, MI). 10 µl of this solution was then mixed with 2 µl 10 mg/ml bovine serum albumin, 33 µl containing (0.5 µg) DMp53 cDNA fragment denatured by quick boiling, 5 µl of 1 mM biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN), and 1 µl of Klenow DNA polymerase (2 U) (Boehringer Mannheim). The mixture was incubated at room temperature overnight and the following components were then added: 1 µl of 1 mg/ml sonicated denatured salmon sperm DNA, 5.5 µl 3 M sodium acetate pH 5.2, and 150 µl

ethanol (100%). After mixing the solution was stored at -70°C for 1-2 hr. DNA precipitate was collected by centrifugation in a microcentrifuge and the pellet was washed once in 70% ethanol, dried in a vacuum, dissolved in 50 µl TE buffer, and stored at -20°C.

(C) Hybridization and staining was performed as follows: 20 µl of the probe added
5 to a hybridization solution (112.5 µl formamide; 25 µl 20x SSC, pH 7.0; 50 µl 50% dextran sulfate; 62.5 µl distilled H₂O) was placed on the squash. A coverslip (22 mm²) was placed on the squash and sealed with rubber cement and placed on the airtight moist chamber overnight at 42°C. Rubber cement was removed by peeling off cement, then coverslip removed in 2x SSC buffer at 37°C. Slides were washed twice 15 min each in 2x SSC buffer
10 at 37°C. Slides were then washed twice 15 min each in PBS buffer at room temperature. A mixture of the following "Elite" solution was prepared by mixing: 1 ml of PBT buffer (PBS buffer with 0.1% Tween 20), 10 µl of Vectastain A (Vector Laboratories, Burlingame, CA), and 10 µl of Vectastain B (Vector Laboratories). The mixture was then allowed to incubate for 30 min. 50 µl of the Elite solution was added to the slide then drained off. 75 µl of the
15 Elite solution was added to slide and a coverslip was placed onto the slide. The slide was incubated in moist chamber 1.5-2 hr at 37°C. The coverslip was then removed in PBS buffer, and the slide was washed twice 10 min each in PBS buffer.

A fresh solution of DAB (diaminobenzidine) in PBT buffer was made by mixing 1 µl of 0.3% hydrogen peroxide with 40 µl 0.5 mg/ml DAB solution. 40 µl of the
20 DAB/peroxide solution was then placed onto each slide. A coverslip was placed onto the slide and incubated 2 min. Slides were then examined under a phase microscope and reaction was stopped in PBS buffer when signal was determined to be satisfactory. Slides were then rinsed in running H₂O for 10 min. and air dried. Finally, slides were inspected under a compound microscope to assign a chromosomal location to the hybridization signal.
25 A single clear region of hybridization was observed on the polytene chromosome squashes which was assigned to cytogenetic bands 94D2-6.

Example 7: Isolation and sequence analysis of a genomic clone for the DMp53 gene

PCR was used to generate DNA probes for identification of genomic clones
30 containing the DMp53 gene. Each reaction (50 µl total volume) contained 100 ng *Drosophila* genomic DNA, 2.5 µM each dNTP, 1.5 mM MgCl₂, 2 µM of each primer, and 1 µl of TAKARA exTaq DNA polymerase (PanVera Corp., Madison, WI). Reactions were set up with primer pair 5'164 & 3'510 (described above), and thermocycling conditions used were as follows (where 0:00 indicates time in minutes:seconds): initial denaturation of

94°C, 2:00; followed by 10 cycles of 94°C, 0:30, 58°C 0:30, 68°C, 4:00; followed by 20 cycles of 94°C, 0:30, 55°C, 0:30, 68°C, 4:00 + 0:20 per cycle. PCR products were then fractionated by agarose gel electrophoresis, ³²P-labeled by nick translation, and hybridized to nylon membranes containing high-density arrayed P1 clones from the Berkeley

5 *Drosophila* Genome Project (University of California, Berkeley, and purchased from Genome Systems, Inc., St. Louis, MO). Four positive P1 clones were identified: DS01201, DS02942, DS05102, and DS06254, and each clone was verified using a PCR assay with the primer pair described above. To prepare DNA for sequencing, *E. coli* containing each P1 clone was streaked to single colonies on LB agar plates containing 25 µg/ml kanamycin,

10 and grown overnight at 37°C. Well-separated colonies for each P1 clone were picked and used to inoculate 250 ml LB medium containing 25 µg/ml kanamycin and cultures were grown for 16 hours at 37°C with shaking. Bacterial cells were collected by centrifugation, and DNA purified with a Qiagen Maxi-Prep System kit (QIAGEN, Inc., Valencia, California). Genomic DNA sequence from the P1 clones was obtained using a strategy that

15 combined shotgun and directed sequencing of a small insert plasmid DNA library derived from the P1 clone DNAs (Ruddy *et al.* Genome Research (1997) 7:441-456). All DNA sequencing and analysis were performed as described before, and P1 sequence contigs were analyzed using the BLAST sequence homology searching programs to identify those that contained the DMp53 gene or other coding regions. This analysis demonstrated that the

20 DMp53 gene was divided into 8 exons and 7 introns. In addition, the BLAST analysis indicated the presence of two additional genes that flank the DMp53 gene; one exhibited homology to a human gene implicated in nephropathic cystinosis (labeled CTNS-like gene) and the second gene exhibited homology to a large family of oxidoreductases. Thus, we could operationally define the limits of the DMp53 gene as an 8,805 bp corresponding the

25 DNA region lying between the putative CTNS-like and oxidoreductase-like genes.

Example 8: Analysis of p53 Nucleic Acid Sequences

Upon completion of cloning, the sequences were analyzed using the Pfam and Prosite programs, and by visual analysis and comparison with other p53 sequences.

30 Regions of cDNA encoding the various domains of SEQ ID Nos 1-6 are depicted in Table I above. Additionally, Pfam predicted p53 similarity regions for the partial TRIB-Bp53 at amino acid residues 118-165 (SEQ ID NO:8) encoded by nucleotides 354-495 (SEQ ID NO:7), and for the partial HELIOp53 at amino acid residues 105-138 (SEQ ID NO:10) encoded by nucleotides 315-414 (SEQ ID NO:9).

Nucleotide and amino acid sequences for each of the p53 nucleic acid sequences and their encoded proteins were searched against all available nucleotide and amino acid sequences in the public databases, using BLAST (Altschul *et al.*, *supra*). Tables 2-6 below summarize the results. The 5 most similar sequences are listed for each p53 gene.

5

TABLE 2 – DMp53

DNA BLAST of SEQ ID NO:1	
GI#	DESCRIPTION
6664917= C019980	<i>Drosophila melanogaster</i> . *** SEQUENCING IN PROGRESS ***, in ordered pieces
5670489=AC008200	<i>Drosophila melanogaster</i> chromosome 3 clone BACR17P04. (D757) RPCI-98 17.P.4 map 94D-94E strain y; cn bw sp, *** SEQUENCING IN PROGRESS***, 70 unordered pieces.
4419483=AI516383	<i>Drosophila melanogaster</i> cDNA clone LD42237 5prime, mRNA sequence
4420516=AI517416	<i>Drosophila melanogaster</i> cDNA clone GH28349 5prime, mRNA sequence
4419333=AI516233	<i>Drosophila melanogaster</i> cDNA clone LD42031 5prime, mRNA sequence
PROTEIN BLAST of SEQ ID NO:2	
GI#	DESCRIPTION
1244764= AA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
1244762= AA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
2828704= AC31133	tumor protein p53 [<i>Xiphophorus helleri</i>]
2828706= AC31134	tumor protein p53 [<i>Xiphophorus maculatus</i>]
3695098= AC62643	DN p63 beta [<i>Mus musculus</i>]

TABLE 3 – CPBp53

DNA BLAST of SEQ ID NO:3	
GI#	DESCRIPTION
6468070= AC008132	<i>Homo sapiens</i> , complete sequence Chromosome 22q11 PAC Clone pac995o6 In CES-DGCR Region
4493931= AL034556	<i>Plasmodium falciparum</i> MAL3P5, complete sequence
3738114= AC004617	<i>Homo sapiens</i> chromosome Y, clone 264,M.20, complete sequence
4150930= AC005083	<i>Homo sapiens</i> BAC clone CTA-281G5 from 7p15-p21, complete sequence
4006838= AC006079	<i>Homo sapiens</i> chromosome 17, clone hRPK.855_D_21, complete sequence
PROTEIN BLAST of SEQ ID NO:4	
GI#	DESCRIPTION
1244764= AA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
1244762= AA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
4530686=AA03817	unnamed protein product [<i>unidentified</i>]

4803651=CAA72225	P73 splice variant [<i>Cercopithecus aethiops</i>]
2370177=CAA72219	first splice variant [<i>Homo sapiens</i>]

TABLE 4 – TRIB-Ap53

DNA BLAST of SEQ ID NO:5	
GI#	DESCRIPTION
5877734=AW024204	wv01h01.x1 NCI_CGAP_Kid3 <i>Homo sapiens</i> cDNA clone IMAGE:2528305 3', mRNA sequence
16555= X65053	<i>A.thaliana</i> mRNA for eukaryotic translation initiation factor 4A-2
6072079=AW101398	sd79d06.y1 Gm-c1009 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1009-612 5', mRNA sequence
6070492=AW099879	sd17g11.y2 Gm-c1012 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1012-2013 5', mRNA sequence
4105775= AF049919	<i>Petunia x hybrida</i> PGP35 (PGP35) mRNA, complete cds.
PROTEIN BLAST of SEQ ID NO:6	
GI#	DESCRIPTION
1244764=AAA98564	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
3273745=AAC24830	p53 homolog [<i>Homo sapiens</i>]
1244762=AAA98563	p53 tumor suppressor homolog [<i>Loligo forbesi</i>]
3695096=AAC62642	N p63 gamma [<i>Mus musculus</i>]
3695080=AAC62634	DN p63 gamma [<i>Homo sapiens</i>]

5

TABLE 5 – TRIB-Bp53

DNA BLAST of SEQ ID NO:7	
GI#	DESCRIPTION
4689085= AF043641	<i>Barbus barbus</i> p73 mRNA, complete cds
4530689= A64588	Sequence 7 from Patent WO9728186
N/A	No further homologies
PROTEIN BLAST of SEQ ID NO:8	
GI#	DESCRIPTION
4689086=AAD27752	p73 [<i>Barbus barbus</i>]
4530686=CAA03817	unnamed protein product [unidentified]
4803651=CAA72225	P73 splice variant [<i>Cercopithecus aethiops</i>]
4530690=CAA03819	unnamed protein product [unidentified]
4530684=CAA03816	unnamed protein product [unidentified]

TABLE 6 – HELIO p53

DNA BLAST of SEQ ID NO:9	
GI#	DESCRIPTION
N/A	No homologies found

PROTEIN BLAST of SEQ ID NO:10	
GI#	DESCRIPTION
2781308= 1YCSA	Chain A, p53-53bp2 Complex
1310770= 1TSRA	Chain A, p53 Core Domain In Complex With Dna
1310771= 1TSRB	Chain B, p53 Core Domain In Complex With Dna
1310772= 1TSRC	Chain C, p53 Core Domain In Complex With Dna
1310960= 1TUPA	Chain A, Tumor Suppressor p53 Complexed With Dna

BLAST analysis using each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous novel amino acids with respect to published sequences indicate the following: 7 amino acid residues for DMp53 and for
 5 TRIB-Ap53, 6 amino acid residues for CPBp53, and 5 amino acid residues for TRIB-Bp53 and HELIOp53.

BLAST results for each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous amino acids for which there are no sequences contained within public database sharing 100% sequence similarity indicate the
 10 following: 9 amino acid residues for DMp53, CPBp5, TRIB-Ap53, and TRIB-Bp53, and 6 amino acid residues for HELIOp53.

Example 9: *Drosophila* genetics

Fly culture and crosses were performed according to standard procedures at 22-25°C
 15 (Ashburner, *supra*). GI-DMp53 overexpression constructs were made by cloning a BclI HincII fragment spanning the DMp53 open reading frame into a vector (pExPress) containing glass multiple repeats upstream of a minimal heat shock promoter. The pExPress vector is an adapted version of the pGMR vector (Hay *et al.*, Development (1994) 120:2121-2129) which contains an alpha tubulin 3' UTR for increased protein stabilization
 20 and a modified multiple cloning site. Standard P-element mediated germ line transformation was used to generate transgenic lines containing these constructs (Rubin and Spradling, *supra*). For X-irradiation experiments, third instar larvae in vials were exposed to 4,000 Rads of X-rays using a Faxitron X-ray cabinet system (Wheeling, IL).

Example 10: Whole-mount RNA *in situ* hybridization, TUNEL, and Immunocytochemistry

In situ hybridization was performed using standard methods (Tautz and Pfeifle, Chromosoma (1989) 98:81-85). DMp53 anti-sense RNA probe was generated by digesting DMp53 cDNA with EcoRI and transcribing with T7 RNA polymerase. For

immunocytochemistry, third instar larval eye and wing discs were dissected in PBS, fixed in 2% formaldehyde for 30 minutes at room temperature, permeabilized in PBS+0.5% Triton for 15 minutes at room temperature, blocked in PBS+5% goat serum, and incubated with primary antibody for two hours at room temperature or overnight at 4°C. Anti-phospho-histone staining used Anti-phospho-histone H3 Mitosis Marker (Upstate Biotechnology, Lake Placid, NY) at a 1:500 dilution. Anti-DMp53 monoclonal antibody staining used hybridoma supernatant diluted 1:2. Goat anti-mouse or anti-rabbit secondary antibodies conjugated to FITC or Texas Red (Jackson ImmunoResearch, West Grove, PA) were used at a 1:200 dilution. Antibodies were diluted in PBS+5% goat serum. TUNEL assay was performed by using the Apoptag Direct kit (Oncor, Gaithersburg, MD) per manufacturer's protocol with a 0.5% Triton/PBS permeabilization step. Discs were mounted in anti-fade reagent (Molecular Probes, Eugene, OR) and images were obtained on a Leica confocal microscope. BrDU staining was performed as described (de Nooij *et al.*, Cell. (1996)87(7):1237-1247) and images were obtained on an Axioplan microscope (Zeiss, Thornwood, NY).

Example 11: Generation of anti-DMp53 antibodies

Anti-DMp53 rabbit polyclonal (Josman Labs, Napa, CA) and mouse monoclonal antibodies (Antibody Solutions Inc., Palo Alto, CA) were generated by standard methods using a full-length DMp53 protein fused to glutathione-S-transferase (GST-DMp53) as antigen. Inclusion bodies of GST-DMp53 were purified by centrifugation using B-PER buffer (Pierce, Rockford, IL) and injected subcutaneously into rabbits and mice for immunization. The final boost for mouse monoclonal antibody production used intravenous injection of soluble GST-DMp53 produced by solubilization of GST-DMp53 in 6M GuHCl and dialysis into phosphate buffer containing 1M NaCl. Hybridoma supernatants were screened by ELISA using a soluble 6XHIS-tagged DMp53 protein bound to Ni-NTA coated plates (Qiagen, Valencia, CA) and an anti-mouse IgG Fc-fragment specific secondary antibody.

Example 12: Functional analysis

The goal of this series of experiments was to compare and contrast the functions of the insect p53s to those of the human p53. The DMp53 was chosen to carry out this set of experiments, although any of the other insect p53s could be used as well.

p53 involvement in the cell death pathway

To determine whether DMp53 can serve the same functions in vivo as human p53, DMp53 was ectopically expressed in *Drosophila* larval eye discs using *glass*-responsive enhancer elements. The *glass*-DMp53 (gl-DMp53) transgene expresses DMp53 in all cells posterior to the morphogenetic furrow. During eye development, the morphogenetic furrow sweeps from the posterior to the anterior of the eye disc. Thus, gl-DMp53 larvae express DMp53 in a field of cells which expands from the posterior to the anterior of the eye disc during larval development.

Adult flies carrying the gl-DMp53 transgene were viable but had small, rough eyes with fused ommatidia (any of the numerous elements of the compound eye). TUNEL staining of gl-DMp53 eye discs showed that this phenotype was due, at least in part, to widespread apoptosis in cells expressing DMp53. Results were confirmed by the detection of apoptotic cells with acridine orange and Nile Blue. TUNEL-positive cells appeared within 15-25 cell diameters of the furrow. Given that the furrow moves approximately 10 cell diameters per hour, this indicated that the cells became apoptotic 2-3 hours after DMp53 was expressed. Surprisingly, co-expression of the baculovirus cell death inhibitor p35 did not block the cell death induced by DMp53 (Miller, J Cell Physiol (1997) 173(2):178-182; Ohtsubo *et al.*, Nippon Rinsho (1996) 54(7):1907-1911). However, DMp53-induced apoptosis and the rough-eye phenotype in gl-DMp53 flies could be suppressed by co-expression of the human cyclin-dependent-kinase inhibitor p21. Because p21 overexpression blocks cells in the G1 phase of the cell cycle, this finding suggests that transit through the cell cycle sensitizes cells to DMp53-induced apoptosis. A similar effect of p21 overexpression on human p53-induced apoptosis has been described.

p53 involvement in the cell cycle

In addition to its ability to affect cell death pathways, mammalian p53 can induce cell cycle arrest at the G1 and G2/M checkpoints. In the *Drosophila* eye disc, the second mitotic wave is a synchronous, final wave of cell division posterior to the morphogenetic furrow. This unique aspect of development provides a means to assay for similar effects of DMp53 on the cell. The transition of cells from G1 to S phase can be detected by BrdU incorporation. Eye discs dissected from wild-type third instar larvae displayed a tight band of BrdU-staining cells corresponding to DNA replication in the cells of the second mitotic wave. This transition from G1 to S phase was unaffected by DMp53 overexpression from the gl-DMp53 transgene. In contrast, expression of human p21 or a *Drosophila* homologue, dacapo (de Nooij *et al.*, Cell (1996) 87(7):1237-1247; Lane *et al.*, Cell (1996) 87(7):1225-1235), under control of *glass*-responsive enhancer elements completely blocked DNA

replication in the second mitotic wave. In mammalian cells, p53 induces a cell cycle block in G1 through transcriptional activation of the p21 gene. These results suggest that this function is not conserved in DMp53.

In wild-type eye discs, the second mitotic wave typically forms a distinct band of cells that stain with an anti-phospho-histone antibody. In *gl*-DMp53 larval eye discs, this band of cells was significantly broader and more diffuse, suggesting that DMp53 alters the entry into and/or duration of M phase.

p53 response to DNA damage

The following experiments were performed to determine whether loss of DMp53 function affected apoptosis or cell cycle arrest in response to DNA damage.

In order to examine the phenotype of tissues deficient in DMp53 function, dominant-negative alleles of DMp53 were generated. These mutations are analogous to the R175H (R155H in DMp53) and H179N (H159N in DMp53) mutations in human p53. These mutations in human p53 act as dominant-negative alleles, presumably because they cannot bind DNA but retain a functional tetramerization domain. Co-expression of DMp53 R155H with wild-type DMp53 suppressed the rough eye phenotype that normally results from wild type DMp53 overexpression, confirming that this mutant acts as a dominant-negative allele *in vivo*. Unlike wild type DMp53, overexpression of DMp53 R155H or H159N using the *glass* enhancer did not produce a visible phenotype, although subtle alterations in the bristles of the eye were revealed by scanning electron microscopy.

In mammalian systems, p53-induced apoptosis plays a crucial role in preventing the propagation of damaged DNA. DNA damage also leads to apoptosis in *Drosophila*. To determine if this response requires the action of DMp53, dominant-negative DMp53 was expressed in the posterior compartment of the wing disc. Following X-irradiation, wing discs were dissected. TUNEL staining revealed apoptotic cells and anti-DMp53 antibody revealed the expression pattern of dominant-negative DMp53. Four hours after X-irradiation, wild type third instar larval wing discs showed widespread apoptosis. When the dominant-negative allele of DMp53 was expressed in the posterior compartment of the wing disc, apoptosis was blocked in the cells expressing DMp53. Thus, induction of apoptosis following X-irradiation requires the function of DMp53. This pro-apoptotic role for DMp53 appears to be limited to a specific response to cellular damage, because developmentally programmed cell death in the eye and other tissues is unaffected by expression of either dominant-negative DMp53 allele. The requirement for DMp53 in the

apoptotic response to X-irradiation suggests that DMp53 may be activated by DNA damage. In mammals, p53 is activated primarily by stabilization of p53 protein.

Although DMp53 function is required for X-ray induced apoptosis, it does not appear to be necessary for the cell cycle arrest induced by the same dose of irradiation. In the absence of irradiation, a random pattern of mitosis was observed in 3rd instar wing discs of *Drosophila*. Upon irradiation, a cell cycle block occurred in wild-type discs as evidenced by a significant decrease in anti-phospho-histone staining. The cell cycle block was unaffected by expression of dominant-negative DMp53 in the posterior of the wing disc. Several time points after X-irradiation were examined and all gave similar results, suggesting that both the onset and maintenance of the X-ray induced cell cycle arrest is independent of DMp53.

p53 in normal development

Similar to p53 in mice, DMp53 does not appear to be required for development because widespread expression of dominant-negative DMp53 in *Drosophila* had no significant effects on appearance, viability, or fertility. Interestingly, *in situ* hybridization of developing embryos revealed widespread early embryonic expression that became restricted to primordial germ cells in later embryonic stages. This expression pattern may indicate a crucial role for DMp53 in protecting the germ line, similar to the proposed role of mammalian p53 in protection against teratogens.

Example 13: p53 RNAi experiments in cell culture

Stable *Drosophila* S2 cell lines expressing hemagglutinin epitope (HA) tagged p53, or vector control under the inducible metallothionein promoter were produced by transfection using pMT/V5-His (Invitrogen, Carlsbad, CA). Induction of DMp53 expression by addition of copper to the medium resulted in cell death via apoptosis. Apoptosis was measured by three different methods: a cell proliferation assay; FACS analysis of the cell population in which dead cells were detected by their contracted nuclei; and a DNA ladder assay. The ability to use RNAi in S2 cell lines allowed p53 regulation and function to be explored using this inducible cell-based p53 expression system.

Preparation of the dsRNA template: PCR primers containing an upstream T7 RNA polymerase binding site and downstream DMp53 gene sequences were designed such that sequences extending from nucleotides 128 to 1138 of the DMp53 cDNA sequence (SEQ ID NO:1) could be amplified in a manner that would allow the generation of a

DMp53-derived dsRNA. PCR reactions were performed using EXPAND High Fidelity (Boehringer Mannheim, Indianapolis, IN) and the products were then purified.

DMp53 RNA was generated from the PCR template using the Promega Large Scale RNA Production System (Madison, WI) following manufacturer's protocols. Ethanol
5 precipitation of RNA was performed and the RNA was annealed by a first incubation at 68°C for 10 min, followed by a second incubation at 37°C for 30 min. The resulting dsRNA was stored at -80°C.

RNAi experiment in tissue culture: RNAi was performed essentially as described previously (<http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html>). On
10 day 1, cultures of Drosophila S2 cells were obtained that expressed pMT-HA-DMp53 expression plasmid and either 15 µg of DMp53 dsRNA or no RNA was added to the medium. On the second day, CuSO₄ was added to final concentrations of either 0, 7, 70 or 700 µM to all cultures. On the fourth day, an alamarBlue (Alamar Biosciences Inc., Sacramento, CA) staining assay was performed to measure the number of live cells in each
15 culture, by measuring fluorescence at 590 nm.

At 7µM CuSO₄, there was no change in cell number from 0 µM CuSO₄ for RNAi treated or untreated cells. At 70 µM CuSO₄, there was no change in cell number from 0 µM CuSO₄ for the RNAi-treated category. However, the number of cells that were not treated with RNAi dropped by 30%. At 700 µM CuSO₄, the number of cells that were treated with
20 RNAi dropped by 30% (as compared with 0µM CuSO₄), while the number of cells that were not treated with RNAi dropped by 70%.

These experiments showed that p53 dsRNA rescued at least 70% of the cells in the p53 inducible category, since some cell loss might be attributable to copper toxicity. Results of these experiments demonstrate that DMp53 dsRNA rescues cells from apoptosis
25 caused by inducing DMp53 overexpression. Thus, this experimental cell-based system represents a defined and unique way to study the mechanisms of p53 function and regulation.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:
 - 5 (a) a nucleic acid sequence that encodes a polypeptide comprising at least 7 contiguous amino acids of any one of SEQ ID NOs 4, 6, 8, and 10;
 - (b) a nucleic acid sequence that encodes a polypeptide comprising at least 7 contiguous amino acids of SEQ ID NO:2, wherein the isolated nucleic acid molecule is less than 15kb in size;
 - 10 (c) a nucleic acid sequence that encodes a polypeptide comprising at least 9 contiguous amino acids that share 100% sequence similarity with 9 contiguous amino acids of any one of SEQ ID NOs 4, 6, 8, and 10;
 - (d) a nucleic acid sequence that encodes a polypeptide comprising at least 9 contiguous amino acids that share 100% sequence similarity with 9 contiguous amino acids of SEQ ID NO 2; wherein the isolated nucleic acid molecule is less than 15kb in size;
 - 15 (e) at least 20 contiguous nucleotides of any of nucleotides 1-111 of SEQ ID NO:1, 1-120 of SEQ ID NO:3, 1-93 of SEQ ID NO:5, and 1-1225 of SEQ ID NO:18;
 - (f) a nucleic acid sequence that encodes a polypeptide comprising an amino acid sequence having at least 80% sequence similarity with a sequence selected from the group consisting of SEQ ID NO:20 and SEQ ID NO:22; and
 - 20 (g) the complement of the nucleic acid of any of (a)-(f).
2. The isolated nucleic acid molecule of Claim 1 that is RNA.
- 25 3. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence has at least 50% sequence identity with a sequence selected from the group consisting of any of SEQ ID NOs:1, 3, 5, 7, 9, 18, 19 and 21.
- 30 4. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a polypeptide comprising an amino acid sequence selected from the group consisting of: RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, KVCTCPGRD, FXCKNSC and FXCQNSC, wherein X is any amino acid.

5. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes at least 17 contiguous amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10.
- 5 6. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a polypeptide comprising at least 19 amino acids that share 100% sequence similarity with 19 amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10.
7. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence
10 encodes a polypeptide having at least 50% sequence identity with any of SEQ ID NOs 2, 4, 6, 8, and 10.
8. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes at least one p53 domain selected from the group consisting of an activation
15 domain, a DNA binding domain, a linker domain, an oligomerization domain, and a basic regulatory domain.
9. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a constitutively active p53.
20
10. The isolated nucleic acid molecule of Claim 1 wherein the nucleic acid sequence encodes a dominant negative p53.
11. A vector comprising the nucleic acid molecule of Claim 1.
25
12. A host cell comprising the vector of Claim 11.
13. A process for producing a p53 polypeptide comprising culturing the host cell of Claim 8 under conditions suitable for expression of the p53 polypeptide and
30 recovering the polypeptide.
14. A purified polypeptide comprising an amino acid sequence selected from the group consisting of:
 - a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10;

- b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
- c) at least 10 contiguous amino acids of a sequence selected from the group consisting of SEQ ID NO:20 and SEQ ID NO:22.

5

15. The purified polypeptide of Claim 14 wherein the amino acid sequence is selected from the group consisting of RICSCPGRD, KICSCPGRD, RVCSCPGRD, KVCSCPGRD, RICTCPGRD, KICTCPGRD, RVCTCPGRD, KVCTCPGRD, FXCKNSC and FXCQNSC, wherein X is any amino acid.

10

16. The purified polypeptide of Claim 14 wherein the amino acid sequence has at least 50% sequence similarity with a sequence selected from the group consisting of SEQ ID NOs 2, 4, 6, 8, and 10.

15

17. A method for detecting a candidate compound or molecule that modulates p53 activity said method comprising contacting a p53 polypeptide, or a nucleic acid encoding the p53 polypeptide, with one or more candidate compounds or molecules, and detecting any interaction between the candidate compound or molecule and the p53 polypeptide or nucleic acid; wherein the p53 polypeptide comprises an amino acid sequence selected from the group consisting of:

20

- a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
- b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10.

25

18. The method of Claim 17 wherein the candidate compound or molecule is a putative pharmaceutical agent.

30

19. The method of Claim 17 wherein the contacting comprises administering the candidate compound or molecule to cultured host cells that have been genetically engineered to express the p53 protein.

20. The method of Claim 17 wherein the contacting comprises administering the candidate compound or molecule to an insect has been genetically engineered to express the p53 protein.
- 5 21. The method of Claim 20 wherein the candidate compound is a putative pesticide.
22. A first insect that has been genetically modified to express or mis-express a p53 protein, or the progeny of the insect, that has inherited the p53 protein expression or mis-expression, wherein the p53 protein comprises an amino acid sequence selected from the group consisting of:
- 10 a) at least 7 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10; and
- b) at least 9 contiguous amino acids that share 100% sequence similarity with at least 9 contiguous amino acids of any one of SEQ ID NOs 2, 4, 6, 8, and 10.
- 15 23. The insect of Claim 22 wherein said insect is *Drosophila* that has been genetically modified to express a dominant negative p53 having a mutation selected from the group consisting of R155H, H159N, and R266T.
- 20 24. A method for studying p53 activity comprising detecting the phenotype caused by the expression or mis-expression of the p53 protein in the first insect of Claim 22.
- 25 25. The method of Claim 24 additionally comprising observing a second insect having the same genetic modification as the first insect which causes the expression or mis-expression of the p53 protein, and wherein the second animal additionally comprises a mutation in a gene of interest, wherein differences, if any, between the phenotype of the first animal and the phenotype of the second animal identifies the gene of interest as capable of modifying the function of the gene encoding the p53 protein.
- 30 26. The method of Claim 24 additionally comprising administering one or more candidate compounds or molecules to the insect or its progeny and observing any changes in p53 activity of the insect or its progeny.

27. A method of modulating p53 activity comprising contacting an insect cell with the isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule is dsRNA derived from a coding region of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, and 9.

5

28. The method of Claim 27 wherein cultured insect cells are contacted with the dsRNA and apoptosis of the cultured cells is assayed.

1/4

Human_p53 MEEPQSDPSVEPPLSQE^TFFSDLWKL^LLPENN^V-----LSP--LPSQAMD
 Xeno_p53 ME-PSSETGMDPPPLSQE^TFFEDLWSL^LP-D-----P^LQTVT
 Squid_p53 MSQGT^S-----PNSQE^TFFNL^LWDSLE^QVTANEY^QIHERGVGYEYHEAEPDQ^TSL^EISA
 CPB_p53 MS-SQSD-----FLPP^DVQNF^LLA^EMEGD-----N^MDN^LN
 Trib_p53A MS-QQS-----QFS^DIIPD^VDK^FLEDH-----GLKDDV
 Trib_p53B -----V-----
 Helio_p53 -----
 Dros_p53 MYISQP-----MSWHKE^STDSEDDSTEVDIK-----ED^IPKTV
 consensus m qs et l le l

Human_p53 D^LM^LS^PDDI--EQWFTEDPGDEAPRMPEA^APRV-----APAPAAP^TPAAPAPAPSW
 Xeno_p53 CR^LLDNLS-----EFPDYPLA^ADMT-----VLQEGLMGNAVPT^VT--
 Squid_p53 YR^IAQDPYGRSESYDLLNPIINQ^IPAPMPI^ADTQNNPLVNHCPEYEDMPVSSTPYSPHDH
 CPB_p53 FFKDEPT-----LNDLNYSN^LNG-----SIVANDD^SKMVHL^IFPG-
 Trib_p53A GR^IIMHEN-----NVHLVND^DGE-----EEKYSNEANYTES^IIFPP-
 Trib_p53B RQYKPPD-----FSHTFHP^IIICSIFQLED^FKFNINQS^SYLSAP^IIFPP-
 Helio_p53 -----
 Dros_p53 EVSGSELT-----TEPMAFLQ^GLN^SGN-----LMQFSQ^SSVLREM^MLQDI
 consensus m p a t v p

Human_p53 PL--SS^SVPS^QKTYQGSYGFR^LGFLH-SGTAKSV^TCTYSPALNK^MFCQ^LAKTC^PVQLWVD
 Xeno_p53 ---SCAVPS^TDD^DYAGKYGLQ^LDFQO-NGTAKSV^TCTYSPALNK^LFCQ^LAKTC^PLV^RVE
 Squid_p53 VQSP^QP^SVPS^NIKYPGEYVFEM^SFAQPSKETKST^TWTYSEKLDK^LYVRMA^TTC^PVRFKTA
 CPB_p53 ---VQ^TS^VPS^NDEYDGPYEF^VDVHP--TVAKN^SWVYST^TLNK^VYMT^GSP^FVDFRVS
 Trib_p53A ---DQ^PTNLGTE^EYPGP^NFSV^LISP--NEQKSPWEYSEKLNK^IFI^GINVK^FVAFSVQ
 Trib_p53B ---SEPLELC^NTE^EYGPPLN^FEV^FDP--NVLNKPWEYSPILNK^IYIDMKHK^FPI^NFSVK
 Helio_p53 -----HEMKCNFSVQ^FNWD
 Dros_p53 QIQAN^TLPKLENHNIGGYCF^SMV^LDE-----PPKSLWMYSIPLNK^LYIRMNKAFNVDVQ^FK
 consensus q svpsq dy g y f l v k tw ys lnkmyv ma fpv f v

FIG. 1A

2/4

Human_p53 STPP-PGTRVRAMAIYKQSQHMTETVRRCPHHERCSDDGLAPPQHLLIRVEGNLR
Xeno_p53 SPPP-RGSI LRATAVYKKSEHVAE VVKRCPPHHERSVEPGE DAAPP SHLMRVEGNLQ
Squid_p53 RPPP-SGCQ LRAMPYIMKPEHVQEVVKRCPPHATAKEHNEKHPAPLHIVRCEHKL-
CPB_p53 HRPP-NPLFI RSTPVYSAQFAQECVYRCNLNHEFSHKESSDGLKEHIRPHIIRCANQYA
Trib_p53A NRPQNLPLY LRATPVFSQTQHFQDLVHRCVGHHPQDQSNKGVAPHIFQHIIRCTNDNA
Trib_p53B KADPERRLFVRVMPMEEDRYVQELVHRCICHEQLTDPTNHNVSEMVAQHIIRCDNNA
Helio_p53 YQKA-PHMFV RSTVVFSDETQAEKRVRCVQHFFHESSTSGIQTEIAKNVLSHSSREIGTQG
Dros_p53 SKMPIQPLNLRVFLCFISND--VSAPVVRCCNHL SVEPLT--ANNAKMRESLLRSENPN
consensus pp lfvRatpvys hvqev RClNHe d s i hiiRcennna

Human_p53 VEYLDDRNT---FRHSVVVPYEP---PEVGS DCTTIHYNYM CNSSCMG-GMNRPRPILTI
Xeno_p53 AYYME DVNS---GRHSVCVPYEG---PQVGT ECTTVLYNYM CNSSCMG-GMNRPRPILTI
Squid_p53 AKYHE DKYS---GRQSVLIPHEM---PQAGSEWVNLVYQFMCLGSCVG-GPNRRPIQLV
CPB_p53 AYLGD KSKN---ERLSVVI PFGI---PQTGESVREIFEFVCKNSCPSPGMNRRAVEII
Trib_p53A LYFGDKNTG---TRLNIVLPLAH---PQVGEDVVKEFFQFVCKNSCPL-GMNRPRPIDVV
Trib_p53B QYFGDKNAG---KRLS-----
Helio_p53 VYYCGKVDM---ADSWYSVLVEF---MRTSSESCSHAYQFSCKNSCAT-GINRRRAIAT
Dros_p53 VYCGNAQKGKISERFSVVVPLNMMSRSVTRSGLTRQTLAFKFCVQNSCTG---RKE TSLV
consensus vyygdk r svvvp e pq gse t ynfmc nscmg gmnrrpi li

FIG.-1B

3/4

Human_p53 ITLEDSSGNLLGRNSFEVVRVCACPGRRDRRTTEEE-NLRKKG---EPHHELPPGSTKRALP
 Xeno_p53 ITLETPQGLLLGRRCFEVVRVCACPGRRDRRTTEED-NYTKK-----RGLKPSG--KRELA
 Squid_p53 FTLEK-DNQVLGRRAVEVRICACPGRRDRKADDEKASLVSK-----PPSPKKNGFPPQRSIV
 CPB_p53 FTLEDNQGTIYGRKTLNVRICSCPKRDKDEKDEKDNANT-----NLPHG-KRRKME
 Trib_p53A FTLEDNKGEVFGRRLVGVRVCSGPCRDKDKDEK-DMESA-----VPPRRKKRKLIG
 Trib_p53B -----
 Helio_p53 FTLEDAMGNIHGRQKVGAR-----
 Dros_p53 FCLEKACGDIYGHVIVHVKICTCPKRRDRIODERQLNSKKRKSVPAAEEDPSKVRRCIA
 consensus ftled g ligr v vrvc cp rdr eek k p g kr l

Human_p53 NNTSSSP-QPKK---KPLDGEYFTLQIRGRERFEFRLENEALELKDQAQAG-----
 Xeno_p53 HPPSSEPLPKKRLVVDDDEIEFTLRIRKGRSRYEMIKKLNDALELQESLDQO-----
 Squid_p53 LTNDITKITPKK---RKIDDECFTLKVRGRNRYEILCKLRDIMELAAARIPAEARLLYKQE
 CPB_p53 KPSKKPMQTQAE---NDTKFTLTIPLVGRHNEQNVLYCHDLMAGEILRN-----
 Trib_p53A NDERRVVPQSS---DNKIFALNTHIPGKKNYLQALKMCQDMLANEILKKQ-----
 Trib_p53B -----
 Helio_p53 -----
 Dros_p53 IKTEDTESNDSRDCDDSAAEWNVSRTPDGDYRLAITCPNKEWLLQSIIEGMIK-----
 consensus k e tl i gr f m kl e l d l

Human_p53 -KEPGG---SRAHSSHLKSKKGQ-----STSRHKKLMFKTEGPDSD-----
 Xeno_p53 -KVTIK---CRKCRDEIKPKKG-----KKLLVKDEQPDSE-----
 Squid_p53 RQAPIGRLTSLPSSSSNGSQDGSRSSTAFSTSDSSQVNSSQNNNTQMVNGQVPHEEETPVT
 CPB_p53 ---IG---NGTEGPKIALN-----KINTLIRESSSE-----
 Trib_p53A ---EQG---GD-DSADKNCYN-----EITVLLNGTAAFD-----
 Trib_p53B -----
 Helio_p53 -----
 Dros_p53 --EAAAEVLRNPQENLRHAN-----KLSLKKRAYELP-----
 consensus g k lmv d

FIG.-1C

4 / 4

Human_p53
Xeno_p53
Squid_p53 KCEPTENTIAQWLTKLGLQAYIDNFQQKGLHNMFQLDEFTLEDLQSMRIGTGHRNKIWKs
CPB_p53
Trib_p53A
Trib_p53B
Helio_p53
Dros_p53
consensus

Human_p53
Xeno_p53
Squid_p53 LLDYRRLSSGTEsqALQHAASNAstLSVGSQNSYCPGFYEVTryTKHTISYL
CPB_p53
Trib_p53A
Trib_p53B
Helio_p53
Dros_p53
consensus

FIG._1D

SEQUENCE LISTING

<110> EXELIXIS, INC

<120> Insect p53 Tumor Suppressor Genes and Proteins

<130> Insect p53 sequences

<140> EX00-015

<141> 2000-03-13

<150> EX99-001

<151> 1999-03-16

<160> 22

<170> PatentIn Ver. 2.1

<210> 1

<211> 1573

<212> DNA

<213> Drosophila melanogaster

<400> 1

```

aaaatccaaa tagtcggtgg ccactacgat tctgtagttt tttgtagcg aatttttaat 60
athtagctc cttccccaac aagatcgctt gatcagatat agccgactaa gatgtatata 120
tcacagccaa tgcgtggca caaagaaagc actgattccg aggatgactc cacggaggtc 180
gatatcaagg aggatattcc gaaaacggtg gaggtatcgg gatcggaatt gaccacggaa 240
cccatggcct tcttgcaagg attaaactcc gggaatctga tgcagttcag ccagcaatcc 300
gtgctgcgcg aaatgatgct gcaggacatt cagatccagg cgaacacgct gccaagcta 360
gagaatcaca acatcggtgg ttattgcttc agcatgggtc tggatgagcc gccaagtct 420
ctttggatgt actcgattcc gctgaacaag ctctacatcc ggatgaacaa ggccttcaac 480
gtggacgttc agttcaagtc taaaatgccc atccaaccac ttaatttgcg tgtgttcctt 540
tgcttctcca atgatgtgag tgctcccggtg gtccgctgtc aaaatcacct tagcgttgag 600
cctttgacgg ccaataacgc aaaaatgcgc gagagcttgc tgcgcagcga gaatcccaac 660
agtgtatatt gtggaaatgc tcagggcaag ggaatttccg agcgtttttc cgtttagtc 720
cccctgaaca tgagccggtc tgtaacccgc agtgggctca cgcgccagac cctggccttc 780
aagttcgtct gccaaaactc gtgtatcggg cgaaaagaaa ctctcttagt cttctgcctg 840
gagaaagcat gcggcgatat cgtgggacag catgttatac atgttaaaat atgtacgtgc 900
cccaagcggg atcgcatcca agacgaacgc cagctcaata gcaagaagcg caagtccgtg 960
ccggaagccg ccgaagaaga tgagccgtcc aagggtgcgtc ggtgcattgc tataaagacg 1020
gaggacacgg agagcaatga tagccgagac tgcgacgact ccgccgcaga gtggaacgtg 1080
tcgcggaacac cggatggcga ttaccgtctg gctattacgt gccccataa ggaatggctg 1140
ctgcagagca tcgagggtcat gattaaggag gcggcggtg aagtcctgcg caatcccaac 1200
caagagaatc tacgtcgcca tgccaacaaa ttgctgagcc ttaagaacg tgcctacgag 1260
ctgccatgac ttctgatctg gtcgacaatc tcccaggat cagatacctt tgaaatgtgt 1320
tgcattctgt gggatatacta catagctatt agtatcttaa gtttgtatta gtcctgttc 1380
gtaaggcgtt taacggtgat attccccctt tggcatgttc gatggccgaa aagaaaacat 1440

```

SEQUENCE LISTING

<110> EXELIXIS, INC

<120> Insect p53 Tumor Suppressor Genes and Proteins

<130> Insect p53 sequences

<140> EX00-015

<141> 2000-03-13

<150> EX99-001

<151> 1999-03-16

<160> 22

<170> PatentIn Ver. 2.1

<210> 1

<211> 1573

<212> DNA

<213> Drosophila melanogaster

<400> 1

```

aaaatccaaa tagtcggtgg ccactacgat tctgtagttt tttgttagcg aatttttaaat 60
atttagcctc cttccccaac aagatcgctt gatcagatat agccgactaa gatgtatata 120
tcacagccaa tgtcgtggca caaagaaagc actgattccg aggatgactc cacggaggtc 180
gatatcaagg aggatattcc gaaaacggtg gaggtatcgg gatcggaatt gaccacggaa 240
cccatggcct tcttgcaggg attaaactcc gggaaatcga tgcagttcag ccagcaatcc 300
gtgctgcgcg aaatgatgct gcaggacatt cagatccagg cgaacacgct gccaagcta 360
gagaatcaca acatcggtgg ttattgcttc agcatggttc tggatgagcc gccaagtct 420
ctttggatgt actcgattcc gctgaacaag ctctacatcc ggatgaacaa ggccttcaac 480
gtggacgttc agttcaagtc taaaatgccc atccaaccac ttaatttgcg tgtgttcctt 540
tgcttctcca atgatgtgag tgctcccggtg gtccgctgtc aaaatcacct tagcgttgag 600
cctttgacgg ccaataacgc aaaaatgcgc gagagcttgc tgcgcagcga gaatcccaac 660
agtgtatatt gtggaaatgc tcagggcaag ggaatttccg agcgtttttc cgttgtagtc 720
cccctgaaca tgagccggtc tgtaaccgcg agtggggtca cgcgccagac cctggccttc 780
aagttcgtct gccaaaactc gtgtatcggg cgaaaagaaa cttccttagt cttctgcctg 840
gagaaagcat gcggcgatat cgtgggacag catgttatac atgttaaaat atgtacgtgc 900
cccaagcggg atcgcatcca agacgaacgc cagctcaata gcaagaagcg caagtcctgtg 960
ccggaagccg ccgaagaaga tgagccgtcc aagggtgcgtc ggtgcattgc tataaagacg 1020
gaggacacgg agagcaatga tagccgagac tgcgacgact ccgccgcaga gtggaacgtg 1080
tcgcggacac cggatggcga ttaccgtctg gctattacgt gcccataa ggaatggctg 1140
ctgcagagca tcgagggcat gattaaggag gcggcggtg aagtcctgcg caatcccaac 1200
caagagaatc tacgtcgcca tgccaacaaa ttgctgagcc ttaagaaacg tgcctacgag 1260
ctgccatgac ttctgatctg gtcgacaatc tcccaggtat cagatacctt tgaaatgtgt 1320
tgcacatctg ggtatacta catagctatt agtatcttaa gtttgtatta gtcottgttc 1380
gtaaggcggt taacggtgat attccccttt tggcatgttc gatggccgaa aagaaaacat 1440

```

ttttatattt ttgatagtat actgttgta actgcagttc tatgtgacta cgtaactttt 1500
 gtctaccaca acaaacatac tctgtacaaa aaagccaaaa gtgaatttat taaagagttg 1560
 tcatattttg caa 1573

<210> 2

<211> 385

<212> PRT

<213> Drosophila melanogaster

<400> 2

Met	Tyr	Ile	Ser	Gln	Pro	Met	Ser	Trp	His	Lys	Glu	Ser	Thr	Asp	Ser
1				5					10					15	
Glu	Asp	Asp	Ser	Thr	Glu	Val	Asp	Ile	Lys	Glu	Asp	Ile	Pro	Lys	Thr
			20					25					30		
Val	Glu	Val	Ser	Gly	Ser	Glu	Leu	Thr	Thr	Glu	Pro	Met	Ala	Phe	Leu
			35				40					45			
Gln	Gly	Leu	Asn	Ser	Gly	Asn	Leu	Met	Gln	Phe	Ser	Gln	Gln	Ser	Val
	50					55				60					
Leu	Arg	Glu	Met	Met	Leu	Gln	Asp	Ile	Gln	Ile	Gln	Ala	Asn	Thr	Leu
65					70				75					80	
Pro	Lys	Leu	Glu	Asn	His	Asn	Ile	Gly	Gly	Tyr	Cys	Phe	Ser	Met	Val
				85				90					95		
Leu	Asp	Glu	Pro	Pro	Lys	Ser	Leu	Trp	Met	Tyr	Ser	Ile	Pro	Leu	Asn
			100					105					110		
Lys	Leu	Tyr	Ile	Arg	Met	Asn	Lys	Ala	Phe	Asn	Val	Asp	Val	Gln	Phe
	115					120					125				
Lys	Ser	Lys	Met	Pro	Ile	Gln	Pro	Leu	Asn	Leu	Arg	Val	Phe	Leu	Cys
	130					135					140				
Phe	Ser	Asn	Asp	Val	Ser	Ala	Pro	Val	Val	Arg	Cys	Gln	Asn	His	Leu
145					150					155				160	
Ser	Val	Glu	Pro	Leu	Thr	Ala	Asn	Asn	Ala	Lys	Met	Arg	Glu	Ser	Leu
				165					170				175		
Leu	Arg	Ser	Glu	Asn	Pro	Asn	Ser	Val	Tyr	Cys	Gly	Asn	Ala	Gln	Gly
			180					185					190		
Lys	Gly	Ile	Ser	Glu	Arg	Phe	Ser	Val	Val	Val	Pro	Leu	Asn	Met	Ser

195	200	205
Arg Ser Val Thr Arg Ser Gly Leu Thr Arg Gln Thr Leu Ala Phe Lys		
210	215	220
Phe Val Cys Gln Asn Ser Cys Ile Gly Arg Lys Glu Thr Ser Leu Val		
225	230	235 240
Phe Cys Leu Glu Lys Ala Cys Gly Asp Ile Val Gly Gln His Val Ile		
245	250	255
His Val Lys Ile Cys Thr Cys Pro Lys Arg Asp Arg Ile Gln Asp Glu		
260	265	270
Arg Gln Leu Asn Ser Lys Lys Arg Lys Ser Val Pro Glu Ala Ala Glu		
275	280	285
Glu Asp Glu Pro Ser Lys Val Arg Arg Cys Ile Ala Ile Lys Thr Glu		
290	295	300
Asp Thr Glu Ser Asn Asp Ser Arg Asp Cys Asp Asp Ser Ala Ala Glu		
305	310	315 320
Trp Asn Val Ser Arg Thr Pro Asp Gly Asp Tyr Arg Leu Ala Ile Thr		
325	330	335
Cys Pro Asn Lys Glu Trp Leu Leu Gln Ser Ile Glu Gly Met Ile Lys		
340	345	350
Glu Ala Ala Ala Glu Val Leu Arg Asn Pro Asn Gln Glu Asn Leu Arg		
355	360	365
Arg His Ala Asn Lys Leu Leu Ser Leu Lys Lys Arg Ala Tyr Glu Leu		
370	375	380

Pro
385

<210> 3
<211> 2600
<212> DNA
<213> Leptinotarsa decemlineata

<400> 3
gtgttttagtt attgttcggg ggctgttttt ttaattaaaa atttcacggg taaatctttg 60
ttgtcttttc tttttctaatt tgtatcagaa tagctttttt aactgtgaaa accggaaggg 120
atgtcttctc agtcagactt tttacctcca gatgttcaaa atttcctctt ggcagaaatg 180

```

gaaggggaca atatggataa tctaaacttt ttcaaggacg aaccaacttt gaatgattta 240
aattattcaa acatccctaa tggatcaata gttgctaata atgattcaaa gatggttcat 300
cttatttttc cgggagtaca aacaagtgtc ccatcaaata atgaatacga tgggccatat 360
gaatttgaag tagatgttca tcccaactgtg gcaaaaaatt cgtgggtgta ctctaccacc 420
ctgaataaag tttatatgac aatgggcagt ccatttcctg tagatttcag agtatcacat 480
cgacccccga acccattatt catcaggagc actcccgttt acagtgtctc ccaatttgct 540
caagaatgtg tttaccggtg cctaaacat gaattctctc ataaagagtc tgatggagat 600
ctcaaggaac acattcgccc tcatatcata agatgtgcca atcagtatgc tgcttactta 660
ggtgacaagt ctaaaaatga acgtctcagc gttgtcatac cattcggtat cccgcagacg 720
gggtactgaaa gtgttagaga aatttttcgaa tttgtttgca aaaattcttg cccaagtcct 780
ggaatgaata gaagagctgt ggaaataata ttcactttgg aggataatca aggaactatc 840
tatggacgca aaacattaaa tgtgagaata tgctcttgtc caaacgtga taaagagaaa 900
gatgaaaagg ataacactgc caacactaat ctgccgcag gcaaaaagag aaaaatggag 960
aagccatcaa agaaacccat gcagacacag gcagaaaatg ataccaaaga gtttactctg 1020
accataccgc tgggtgggtcg acataatgaa caaatgtgt tgaagtattg ccatgatttg 1080
atggccgggg aaatcctgcg aaatatcggc aatgggtactg aagggccgta caaatagct 1140
ttaaacaaaa taaacacgtt gatacgtgaa agttccgagt gaccttatca attctatgta 1200
tatttcttat acaattccat tttcatattt ccatttgata ataagaaaca ttttagcacc 1260
ttttaatcct acactgcagg gaagtcaata tttctttagt tttttgcatg atattgtttg 1320
ttataacatt ttttttttca acaacagggtg acttgatttt tgtaagggtat ctcatatttt 1380
atgtttaaga cctaaaacac gaaacaaaaa acatgaatgg tcattgaatt tggctcgata 1440
atcaatccaa tgttctttaa agtaatatcg acctgttcac aacttttggt atgcactgaa 1500
tggcttttta ttattattat ttttcagcat tgtacatcat acttgcatag tttcagtttt 1560
aaatttttca aatgtttcat ttattttcat tcttacacct gaacttggat tttggacaca 1620
tggctttcac aatgttctat cacgaacagt atgataagcc aaagtaagag ttgataatag 1680
ttcatattaa tatctattgt aacaccgact attgttatat aaatagtcgt ttttttgta 1740
cttttcttgc tttattttat acacttgagt caagtgtagt cagtacattg actatgctgg 1800
aaaacctgtt ttgagtttat ttttacttac attcagttct catcattaga aattgtttat 1860
tttttggtgt caatatttac gaaaaatggt gcaatactat aataggaaca ttaataaagt 1920
aacttgaaag catagagggt gtgaattttg tttttgatca actttttgaa atttatgcgc 1980
cattctataa gccagttttt tttgataaat tcaaaattca cgaataggta tcaacctgat 2040
tgcagtctta ttctatgttt gtcctaaagc aggtctctat aaaacttctc taaaagttgt 2100
gcagagcaaa taacaaataa ttttttaatg gattatatca attcatgaac tggtttaatt 2160
gaaagagtag attattctat tgggttcaca aaaatataaa taatgtgtta ctatctggat 2220
catttgtttt ttttctattg agctatatat tgtcattgta ttgttgaaact ttccctaaat 2280
cccagtgcc tagtcgacga tcgggtctgc tcccatccat caattattcg aaatctcatt 2340
tatttttaaag actgaggacg ggggtgggact gtcagtgtat ctgtttaatg agaaccatct 2400
tgtactagga ttgatatgtg aatctatgag taggtgcatt tttatatata tatctttatg 2460
tttattttagt attattgtac aggttatgta ctctagtggga agaatacata acctaatat 2520
tatatatgtt cgtaaatata caaatttttt acgtttttta aatatatttt ctaaatattc 2580
aacaacaaaa aaaaaaaaaa 2600

```

<210> 4

<211> 354

<212> PRT

<213> Leptinotarsa decemlineata

<400> 4

Met Ser Ser Gln Ser Asp Phe Leu Pro Pro Asp Val Gln Asn Phe Leu
 1 5 10 15

Leu Ala Glu Met Glu Gly Asp Asn Met Asp Asn Leu Asn Phe Phe Lys
 20 25 30

Asp Glu Pro Thr Leu Asn Asp Leu Asn Tyr Ser Asn Ile Leu Asn Gly
 35 40 45

Ser Ile Val Ala Asn Asp Asp Ser Lys Met Val His Leu Ile Phe Pro
 50 55 60

Gly Val Gln Thr Ser Val Pro Ser Asn Asp Glu Tyr Asp Gly Pro Tyr
 65 70 75 80

Glu Phe Glu Val Asp Val His Pro Thr Val Ala Lys Asn Ser Trp Val
 85 90 95

Tyr Ser Thr Thr Leu Asn Lys Val Tyr Met Thr Met Gly Ser Pro Phe
 100 105 110

Pro Val Asp Phe Arg Val Ser His Arg Pro Pro Asn Pro Leu Phe Ile
 115 120 125

Arg Ser Thr Pro Val Tyr Ser Ala Pro Gln Phe Ala Gln Glu Cys Val
 130 135 140

Tyr Arg Cys Leu Asn His Glu Phe Ser His Lys Glu Ser Asp Gly Asp
 145 150 155 160

Leu Lys Glu His Ile Arg Pro His Ile Ile Arg Cys Ala Asn Gln Tyr
 165 170 175

Ala Ala Tyr Leu Gly Asp Lys Ser Lys Asn Glu Arg Leu Ser Val Val
 180 185 190

Ile Pro Phe Gly Ile Pro Gln Thr Gly Thr Glu Ser Val Arg Glu Ile
 195 200 205

Phe Glu Phe Val Cys Lys Asn Ser Cys Pro Ser Pro Gly Met Asn Arg
 210 215 220

Arg Ala Val Glu Ile Ile Phe Thr Leu Glu Asp Asn Gln Gly Thr Ile
 225 230 235 240

Tyr Gly Arg Lys Thr Leu Asn Val Arg Ile Cys Ser Cys Pro Lys Arg
 245 250 255

Asp Lys Glu Lys Asp Glu Lys Asp Asn Thr Ala Asn Thr Asn Leu Pro
 260 265 270

His Gly Lys Lys Arg Lys Met Glu Lys Pro Ser Lys Lys Pro Met Gln
 275 280 285

Thr Gln Ala Glu Asn Asp Thr Lys Glu Phe Thr Leu Thr Ile Pro Leu
 290 295 300

Val Gly Arg His Asn Glu Gln Asn Val Leu Lys Tyr Cys His Asp Leu
 305 310 315 320

Met Ala Gly Glu Ile Leu Arg Asn Ile Gly Asn Gly Thr Glu Gly Pro
 325 330 335

Tyr Lys Ile Ala Leu Asn Lys Ile Asn Thr Leu Ile Arg Glu Ser Ser
 340 345 350

Glu Trp

<210> 5

<211> 1291

<212> DNA

<213> *Tribolium castaneum*

<400> 5

acgcgtccgg ccaacttaac ctaaaaattht gtttttcgatg cctactagat ttaaaaacaa 60
 ttgattcaaa tcgtggattt ttattattta aatcatgagc caacaaagtc aatttttcgga 120
 catcattcct gatgttgata aatttttggga agatcatgga ctcaaggacg atgtgggaag 180
 aataatgcac gaaaacaacg tccatttagt aaatgacgac ggagaagaag aaaaatactc 240
 taatgaagcc aattacactg aatcaatttt ccccccgac cagcccacaa acctaggcac 300
 tgaggaatac ccaggccctt ttaattttctc agtcttgatc agccccaacg agcaaaaatc 360
 gccctgggag tattcggaaa aactgaacaa aatattcatc ggcatcaacg tgaaattccc 420
 cgtggccttc tccgtgcaaa accgccccca gaacctgccc ctctacatcc gcgccacccc 480
 cgtgttcagc caaacgcagc acttccaaga cctggtgcac cgctgcgtcg gccaccgcca 540
 cccccaagac cagtccaaca agggcgtcgc cccccacatt ttccagcaca ttattagggtg 600
 caccaacgac aacgccctat actttggcga taaaaacaca gggacgagac tcaacatcgt 660
 cctgcctttg gcccaccccc aggtggggga ggacgtggtc aaggagttht tccagtttgt 720
 gtgcaaaaac tcctgcccctt tggggatgaa tcggcgcccg attgatgtcg ttttcaccct 780
 ggaggataat aagggggagg ttttcgggag gaggttggtg ggggtgaggg tgtgttcgtg 840
 tccgaagcgt gacaaggaca aggaggagaa ggacatggag agtgctgtgc ctccaaggag 900
 gaagaagagg aagttgggga atgatgagcg aagggttggtg ccacagggga gctccgataa 960
 taaaatattt gcgttaataa ttcattatcc tggcaagaag aattatttac aagccctcaa 1020
 gatgtgtcaa gatatgctgg ctaatgaaat tttgaaaaaa caggaacaag gtggcgacga 1080
 ttctgctgat aagaactggtt ataatgagat aactgttctc ttgaacggca cggccgcctt 1140

tgattagttt atttctatat ttaattttat actttgtact tatgcaatat tccagtttac 1200
 ttttgtaata tttttattaa taaatttcta cgttttaaaa aaaaaaaaaa aaaaaaaaaa 1260
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa a 1291

<210> 6

<211> 350

<212> PRT

<213> Tribolium castaneum

<400> 6

Met Ser Gln Gln Ser Gln Phe Ser Asp Ile Ile Pro Asp Val Asp Lys
 1 5 10 15

Phe Leu Glu Asp His Gly Leu Lys Asp Asp Val Gly Arg Ile Met His
 20 25 30

Glu Asn Asn Val His Leu Val Asn Asp Asp Gly Glu Glu Glu Lys Tyr
 35 40 45

Ser Asn Glu Ala Asn Tyr Thr Glu Ser Ile Phe Pro Pro Asp Gln Pro
 50 55 60

Thr Asn Leu Gly Thr Glu Glu Tyr Pro Gly Pro Phe Asn Phe Ser Val
 65 70 75 80

Leu Ile Ser Pro Asn Glu Gln Lys Ser Pro Trp Glu Tyr Ser Glu Lys
 85 90 95

Leu Asn Lys Ile Phe Ile Gly Ile Asn Val Lys Phe Pro Val Ala Phe
 100 105 110

Ser Val Gln Asn Arg Pro Gln Asn Leu Pro Leu Tyr Ile Arg Ala Thr
 115 120 125

Pro Val Phe Ser Gln Thr Gln His Phe Gln Asp Leu Val His Arg Cys
 130 135 140

Val Gly His Arg His Pro Gln Asp Gln Ser Asn Lys Gly Val Ala Pro
 145 150 155 160

His Ile Phe Gln His Ile Ile Arg Cys Thr Asn Asp Asn Ala Leu Tyr
 165 170 175

Phe Gly Asp Lys Asn Thr Gly Thr Arg Leu Asn Ile Val Leu Pro Leu
 180 185 190

Ala His Pro Gln Val Gly Glu Asp Val Val Lys Glu Phe Phe Gln Phe

195	200	205
Val Cys Lys Asn Ser Cys Pro Leu Gly Met Asn Arg Arg Pro Ile Asp		
210	215	220
Val Val Phe Thr Leu Glu Asp Asn Lys Gly Glu Val Phe Gly Arg Arg		
225	230	235 240
Leu Val Gly Val Arg Val Cys Ser Cys Pro Lys Arg Asp Lys Asp Lys		
	245	250 255
Glu Glu Lys Asp Met Glu Ser Ala Val Pro Pro Arg Arg Lys Lys Arg		
	260	265 270
Lys Leu Gly Asn Asp Glu Arg Arg Val Val Pro Gln Gly Ser Ser Asp		
	275	280 285
Asn Lys Ile Phe Ala Leu Asn Ile His Ile Pro Gly Lys Lys Asn Tyr		
	290	295 300
Leu Gln Ala Leu Lys Met Cys Gln Asp Met Leu Ala Asn Glu Ile Leu		
305	310	315 320
Lys Lys Gln Glu Gln Gly Gly Asp Asp Ser Ala Asp Lys Asn Cys Tyr		
	325	330 335
Asn Glu Ile Thr Val Leu Leu Asn Gly Thr Ala Ala Phe Asp		
	340	345 350

<210> 7

<211> 508

<212> DNA

<213> Tribolium castaneum

<400> 7

```

gtacgacaat acaaaccgcc cgatttttcc cacactttcc acccaataat ttgctcaatt 60
ttccagttgg aagacttcaa attcaacatc aaccaaagct cgtacctctc agccccatt 120
ttcccccca gcgagccgct cgagctgtgc aacaccgagt accccggccc cctcaacttc 180
gaggtgtttg tggaccccaa cgtgctcaaa aaccctggg aatactcccc aattctcaac 240
aaaatttaca tcgatatgaa acacaaattc cggattaatt tcagcgtgaa gaaggccgat 300
cctgagcgca ggctttttgt cagagttatg cggatgtttg aggaagacag atatgtgcaa 360
gaattggtgc ataggtgcat ctgtcacgaa caattgacag atccgaccaa tcacaacgtt 420
tcggaaatgg tggctcagca catcattcgg tgtgataaca acaatgctca gtatttcggg 480
gataagaacg ctgggaagag actgagta 508

```

<210> 8

<211> 169

<212> PRT

<213> Tribolium castaneum

<400> 8

Val Arg Gln Tyr Lys Pro Pro Asp Phe Ser His Thr Phe His Pro Ile

1

5

10

15

Ile Cys Ser Ile Phe Gln Leu Glu Asp Phe Lys Phe Asn Ile Asn Gln

20

25

30

Ser Ser Tyr Leu Ser Ala Pro Ile Phe Pro Pro Ser Glu Pro Leu Glu

35

40

45

Leu Cys Asn Thr Glu Tyr Pro Gly Pro Leu Asn Phe Glu Val Phe Val

50

55

60

Asp Pro Asn Val Leu Lys Asn Pro Trp Glu Tyr Ser Pro Ile Leu Asn

65

70

75

80

Lys Ile Tyr Ile Asp Met Lys His Lys Phe Pro Ile Asn Phe Ser Val

85

90

95

Lys Lys Ala Asp Pro Glu Arg Arg Leu Phe Val Arg Val Met Pro Met

100

105

110

Phe Glu Glu Asp Arg Tyr Val Gln Glu Leu Val His Arg Cys Ile Cys

115

120

125

His Glu Gln Leu Thr Asp Pro Thr Asn His Asn Val Ser Glu Met Val

130

135

140

Ala Gln His Ile Ile Arg Cys Asp Asn Asn Asn Ala Gln Tyr Phe Gly

145

150

155

160

Asp Lys Asn Ala Gly Lys Arg Leu Ser

165

<210> 9

<211> 433

<212> DNA

<213> Heliothis virescens

<400> 9

gcacgagatg aagtgcact ttagcgtgca attcaactgg gactatcaga aggcgccgca 60

tatgttcgtg cggctctaccg tcgtgttctc cgatgaaacg caggcggaga agcgggtcga 120

acgatgtgtg cagcatttcc atgaaagctc cacttctgga atccaaacag aaattgccaa 180

```

aaacgtgctc cactcgtccc gggagatcgg taccagggc gtgtactact gcgggaaggt 240
ggacatggca gactcgtggt actcagtgt ggtggagttt atgaggacca gctcggagtc 300
ctgctcccat gcgtaccagt tctcctgcaa gaactcttgt gcaaccggca ttaataggcg 360
ggctattgcc attattttta cgctggaaga tgctatgggc aacatccacg gccgtcagaa 420
agtaggggagc agg                                         433

```

<210> 10

<211> 144

<212> PRT

<213> *Heliothis virescens*

<400> 10

```

His Glu Met Lys Cys Asn Phe Ser Val Gln Phe Asn Trp Asp Tyr Gln
  1              5              10              15

```

```

Lys Ala Pro His Met Phe Val Arg Ser Thr Val Val Phe Ser Asp Glu
          20              25              30

```

```

Thr Gln Ala Glu Lys Arg Val Glu Arg Cys Val Gln His Phe His Glu
      35              40              45

```

```

Ser Ser Thr Ser Gly Ile Gln Thr Glu Ile Ala Lys Asn Val Leu His
      50              55              60

```

```

Ser Ser Arg Glu Ile Gly Thr Gln Gly Val Tyr Tyr Cys Gly Lys Val
      65              70              75              80

```

```

Asp Met Ala Asp Ser Trp Tyr Ser Val Leu Val Glu Phe Met Arg Thr
          85              90              95

```

```

Ser Ser Glu Ser Cys Ser His Ala Tyr Gln Phe Ser Cys Lys Asn Ser
      100              105              110

```

```

Cys Ala Thr Gly Ile Asn Arg Arg Ala Ile Ala Ile Ile Phe Thr Leu
      115              120              125

```

```

Glu Asp Ala Met Gly Asn Ile His Gly Arg Gln Lys Val Gly Ala Arg
      130              135              140

```

<210> 11

<211> 26

<212> DNA

<213> *Drosophila melanogaster*

<400> 11
ccatgctgaa gcaataacca ccgatg 26

<210> 12
<211> 30
<212> DNA
<213> Drosophila melanogaster

<400> 12
ggaacacacg caaattaagt gggtggatgg 30

<210> 13
<211> 23
<212> DNA
<213> Drosophila melanogaster

<400> 13
tgatTTTTgac agcggaccac ggg 23

<210> 14
<211> 28
<212> DNA
<213> Drosophila melanogaster

<400> 14
ggaagtttct tttcgccoga tacacgag 28

<210> 15
<211> 27
<212> DNA
<213> Drosophila melanogaster

<400> 15
ggcacaaaga aagcactgat tccgagg 27

<210> 16
<211> 28
<212> DNA
<213> Drosophila melanogaster

<400> 16
ggaatctgat gcagttcagc cagcaatc 28

<210> 17

<211> 23

<212> DNA

<213> *Drosophila melanogaster*

<400> 17

ggatcgcatc caagacgaac gcc

23

<210> 18

<211> 27425

<212> DNA

<213> *Drosophila melanogaster*

<400> 18

tagccactcg ctagtttata gttcaagggtg aacatacgtg agagttttgt ggcactggac 60
tggaaatagg ctgctagtc tttgtgttcg gccatagcgt taaaaattta agcgaacgcc 120
agtcgtcctg cgcccatggt gctgcaacat tctggcttcg tgtcatgcc ctgaatgttt 180
cacattattt aacccccctt attttttttt tttgtgtggc actggccaaa ggtccaaagg 240
ggcgacatgc tgcaggggcg tggcctgcag ctgcttgcaa cgggcaatta ttgcgcagtt 300
attgcatgtc gtgtgcaatg cctatgaatt attacgtata cacagtgtgt cctcggaat 360
aacgaaagtc cgggaggggg cggggcggtg ttcattgctgc agttgcccc aaattcaacg 420
aaattgctac agttttttatt tgtaatgact gggcatggta agttaatatg attcttcata 480
ctgattaagt gctttttgta cttttttaat tattcaagta aaaatattaa tttgtgtttc 540
atgggacttt ttgtagtagt taccctacta ctacattaaa cattaatttc aaagaagtag 600
atatacgagt aaatgggcaa tatgaaaatt tgaaaaaggt aaagcttatg atactaacta 660
atgccaatg aaaactagga gtatgataat aatatgaaga tagccaccca ggctatccca 720
aaatcgcat caaatccaat ggtgttcatt aaattaggta atcgcatgtg cccttatgtc 780
aaccatatcg ccgctcaacc aagtcatttc ggctcgctgag gcaatcgaga tatggggcgc 840
caccgacctt ggccaacatg ctccacattg ggctccaagt ggcaaccgca aaggctcacgc 900
acagttcgcc attgcgaatc gcatactgcc aatggaaact acattgcgta tctgggtggc 960
ctttgatggc gctctaatta aaggctacct gccactaatt agtgatagac aatcgtcggg 1020
ggagttcggg tggcatcggt ggcaggcact taaccacaaga cagggggggc aactggcatt 1080
ggatggcgt ttttgaattc gtatgtcgga agcagtcgat gcagggttg gggggatgga 1140
aacaatggt gtcaacgcc aaaccactga actgttaaaa gtgccattga atccaacaag 1200
gatgctgggc gcaactgtgc aacctaaca actgtcgga agacagcagc aacatgggca 1260
tgcattggtt gatactggga gtctgttcga tggatccac ttgaaccgaa ccgtactgaa 1320
ccgtgccccg gccagatgag gcgccccacc caacgccact cttgaaaacc ccaagccctt 1380
tgcacgcgt aaatagtttt gtttattgca cattgaaacc gagccagcga gcaattccgg 1440
tggctgctcc gcgcgcgaca cactccagcg atctaatacag caatctcgac gacgaccggg 1500
ctgacatggg gtttctcata cgctcgggta gacgcgacgt cgacgctga tcgaatattt 1560
tcccaatgca ctggcagaaa atgtgtggaa gtgtgagatt aagctcataa attagtagtg 1620
cacttaatgt ggaaaatatt agaaacaaca gtgaacagtt gattggttct cttataaatt 1680
ttattaatta ttgaacattt gaagaaagat attgattaaa tcaacttttg atgtatacat 1740
atatataaaa aagtatatga tgactttcat gttgagaggt cataactttg taatgatatt 1800
ggttctagtc atcatttcgt gaaacagctg tgcaagcatt cgattatatg tggatatgaa 1860


```

tttatttggg ttaatatatt tttcgcagtg tactgcttct gctgcgtcac ttcacattcg 1920
tatcattttac atacgcagca ctgcggagtg agtcgctgag tacctggcgc tctgggggtct 1980
ctgggatctc tgggcttggg gatggatctc cactcgatga tctctccgcc tgggagccca 2040
gatcatcgtc tgctatttgc aagtcgagag tcgcgcgagt cggacgtaca atcgcccgag 2100
cggaatcaag tgtgataaaa gtgaacagaa ctttagccaa gtgcatttgg ctaatggaag 2160
tggtggcaaa agtcaaagcc acacgttata ctcgaattta aaaâcaata aataatgcat 2220
aagcaggcga gtttgaagta attagcacia cgatgatgct ggccggccaac tgaccacat 2280
cgggaaatcg ctctaattca tatttgttgt cgagtgggcc aggataacag gataacagga 2340
tactgctggc tcatattgcat ttgcataat gcaaatagtt cgatctgcag gcgattgagt 2400
gaccgaaagt gttggactgt gccaaataca taaccagcta acgggcaaaa agccactgaa 2460
taaattggccc ttgttactcg gttcgtgtaa tgcgtctacg agtttagccc gtgttctgac 2520
cgagaatcaa ttaaaattta ttgcacgagc atgccaaaca attcgcggtt gcagccacaa 2580
aaacgcactc gaaaaacaat gccaccactc caatcacttg tgaccgcccc ccggtatgc 2640
aaattagcca ttgcagcgat tttgctaatt ctccagctaa acgctagtgg tgagttctca 2700
gttggtcaat atatatatat gtatatatat gaaatatgaa aaatcggaâa acccctttgc 2760
aaacattgct ccgcgcttag ctcatgatga tgccaattcc gagagcgttt tgaagatgca 2820
ctcgccattt gcattcaaaa gccaaagcga taaatggaga agcaaaacca aaactgcata 2880
gatcaattta caagtcggca aaggggttta ctgcgtgcat gtgcattgca gctgctatta 2940
tagatttatt tattggcaaa caccctgaga acgagtttca ttggggggcc taagtgggag 3000
aatgacctac acaggaaagt gctcttaact aagcaactaa cttctggaaa agcggaagt 3060
gagagattaa gtactatctt atagatatgc cagaatatca aaaaagtatc taccagatac 3120
cttgaaagat ctctgcatat ctcaattgca attcatgata agtttgtaa gttacgtttt 3180
ttaatttcca attcaacctt tcaattagtt aataacgcca atctcagaca ttctaaacc 3240
ccctccctac ttaagggtaa atcccgatga tgcttgattg attttctcat tgctcagcta 3300
tgcataaaaa tatcatatta attgatgagc acgagcttag ctaccagaat tgaaatccat 3360
atgactgctc ggcaatttga aaaatgcgtt ggttcccagt catgcgcac ccgttggatt 3420
gaaaccacaa ttcatggcat tccgttctgc ccccagttg cgctgctgct caagtgtccg 3480
ttgcaccagt tgcagctgca gaagatcgtc ggattccggc caccgctgga gtatctgaat 3540
gcggataatc ggatctacgg accggaaatg gtgagcaact tcaagactcg caacggccaa 3600
caggaacttc cggtcagcca ggtgtgctgg cgcactgca acgaggatcc cgattgcatt 3660
gcctatgtcc atctgctgga cacggacgag tgccatggct actcgtactt cgagcgaacc 3720
tcgcgctatc tggccatttc gggatgaactg cctctggtgg cagacggcga ggccgtcttc 3780
tacgaaaaga cctgcctccg aggtgagtaa ttctccagcc aaacctccgg aagtggccgt 3840
gatccgctc taatccattc cgacctgca gttcccgatg cgtgccgtgg gcgtctctgg 3900
gcactgacca aaatccccgg cagcacgctg gtctaccaca gcaagaagac catttcgacg 3960
ctggtcacgc ggcgtgagtg cgccgagcgc tgcttcttcg aaaccagtt ccgatgcctc 4020
tccgcctcct ttgcgcccct ctatcggaac aatcgtgagc ggtaattgac tatttgttgt 4080
ttgttgtttg ctatttgggt gtttgttgtt gtcgggtgtc agtgggtggg tgttgtagt 4140
gctggctgcc ggacaaatga atagcttttg ttgtgcattt ttaatgcatg gtcgagactt 4200
ttcgccggat tatgacatca ctccgaggat ggtgatggga taggttagga ctattcaaca 4260
atgtgtagca agctaataat atgataatat gatattataa tacgaaagaa agatatatcc 4320
agaagacatc atcttttcga agctatgttc ttttccaaac aaatttttac aaaataagat 4380
aagtattttt gaaaagtgag atcatcagca atcatctaga ttttcttaa ctcaagtata 4440
tatcgaattc ttctgaaata accgaactga cttggctata atcgacacat catcgtttag 4500
aagttaataa agcaaccttt aaccctcctc tttcgtagct tccgcggcga ggccggctct 4560
ggccagcgtc cgtctccccg cctcggcaga tgtatgctga gcgacaggga caagaccgtc 4620
cagccggacg ctttcgcgc ggctccatac gacgaggagt acatggagaa ccagtgccac 4680
gaacgggcca tcgaaagtga caactgttcc tacgagctgt acgccaacag cagtttcatc 4740

```

tatgcgagg ccaggtatattt gggcctctcc caaaaagagg tgtgtccgcc gcgcttcgga 4800
 tgctcgcat tatgattgta atcgaaatgg atgggggggc ggatgattga ttgatggctt 4860
 ctacctccgt attgcagtgt caggcgatgt gctcccacga ggcgaagttc tactgccagg 4920
 gtgtctcctt ctactatgta aaccaactct cgctgtccga gtgtctcctc cactcggagg 4980
 acattgtatc cctgggtccg cgaagcctga agctccgtga aaactcggtg tacatgcgga 5040
 gggatcaagt cctggatggt aagatcttct ggggatgtgg tatgctcaat cttaatcgat 5100
 tccttattcc gcagtcggg ttttttgcac ccgcatgag atgaccatta agtacaatcc 5160
 caaggactgg ttcgtcggca agatctatgc cagcatgcac tccaaggact gcctggccag 5220
 aggatcgggc aatgggagtg ttctgtgac gctccagatc ggcagcgagg taaaggagaa 5280
 ccgctgtggc atcctgcgtg cctacgaaat gacacaggaa taccaaaggt aagatgaagt 5340
 ccaatgtcca gtccattttt ttaattatat catttgcatt atttagaacg ttcatatctg 5400
 ctctggtggt catccaaaac aatccaaatg tgcaaaccga gggcgaccgg ctcatcaagg 5460
 ttggctgtat acagagcaat gccaccacat cgctgggctg ttcgggttcgg gacagcagt 5520
 tggatagctc agagcctgtg ccagcgcca ttgactgga gtctcattg gagtacacag 5580
 aacagtgaat gtattcttaa tagaatccct caaaatgctt aattctatca caatcgatac 5640
 ctgcagcatg ttcccacacg aggggtgtgg tctactacaac agcagcactg ggccccatcc 5700
 gcacccagc atctcgcttc agattttggga tctatcccac cagcacgaga ccaacgacgt 5760
 gcagattgga cagaacctgg aactacagat tgtggcggag tacagcccac agcagttggc 5820
 agagcacatg gaggttgcagc tggcaccact acccgacttt cgtgctacct cgctgggtggc 5880
 caagacagcg gacaatgaga actttgtgct gctgatcgac gagcaggagat gtcccacaga 5940
 tgccagtgtg tttcccgctt tggaaagggg acacacagcc agcaggagca tgttgcgcg 6000
 tcgcttccat gccttcaagt tctcaggaac ggccaacgta agcttcgatg taaagattcg 6060
 cttctgcgtg gagcgtgct cgcccagcaa ttgtattagt tcatcctggc aacggagaag 6120
 ggcacaggct gaccaaccag atcgtagacc ggaagaccta cgagttcaga acccgtgta 6180
 catctccacg gtggtggatg tggctccgca accagacaac tttaccagat cgcaggagga 6240
 attgcccctc aactacaata tccgggtgca cggctccggac cagagcaaca ccaatagtta 6300
 tctgtacggc gagcggggag tgctgtcat tgctggcata gacgaccgcg tgcacctgga 6360
 taacgtttgc atcaaccaga gcctgtgat tgcactgttc atcttctggc tgatctgtca 6420
 agttgccttg ctcttcggct gtggaatggg gctgcagcgc taccgccggc tggccaagct 6480
 cgaggatgag cgacgcaggc tgcacgagga gtacctggag gcgaggagag tccactgggc 6540
 ggatcaaggc ggatacacac tctaattgac ggctggaacg caatgcgtat aaaatgcac 6600
 ttaatttaat aaacataaat ctaacataaa tctaacaaat gtttgcaacc gaggataagt 6660
 tcaggagttc ttcttgggat ggtagtgctc ccacttgca tggttttagc aattgaaatc 6720
 cgggcagtgg tgagcattt tgcgcaaata gtcggacaac ttgagcagct cgggtgtccg 6780
 gccacggttg agatgagcct gacggaatgg cgggatcttt aggcggact ttgggttcat 6840
 aaggaagttg cgacggatgt catcaaact gatagtgttg ctcgagttgt attgcttgta 6900
 cagggccagc attacaccaa gcggctttac gtccaccaca ccgctcctc gcacatgaac 6960
 tgatatcatg gcgtggagt ccagatagaa catcaccttg tagttatcgt tactggccac 7020
 gccacgagg cgcatctttt cctcgatcca gcgcatgctg gtggcggacc agatgacaat 7080
 gtcgtagtcc tcgtaggcgg aagtcagaaa ctcgtgcaga tacggacgca ttagctccg 7140
 gcctgtttca gcaggcgatc ggtgatcgaa tagggatatag tctatgtcca ggacaagcag 7200
 cttcttgccc tcacgcggcg gcgctaactc cttgatcttg tagtctcgca cagcagctg 7260
 cacttggtcc aaatagacgg cggagtgtc caccgactct tcgcttcat cggcgtcatc 7320
 gaagtcgtcg accacttcgc caatattatc gggcaggctg cagcactcct cgatatcggc 7380
 ctctgtggag cccaccatca taagcttaaa gttgggcttc agctccaaag cgctgatctt 7440
 cacattgtcg gctgctgtct ttctgcaag tcattggatc ttaaaactga aatatccga 7500
 agcctaggag tgcacgcac ctttgtactt cagggtgagc agcttttgac gttccggacg 7560
 cacctgtgtc ttgcggaata tctcgtgacg cagcacttcc acggtgtcct ggtcgggtgag 7620

```

gtccaccggg tactccttac cactccattt tacaatcact accacttctt tgacctccat 7680
cttagctggg ttctattccg ctattaattt atcacaccat atatggtaat gtatgtttgt 7740
tggatagaat ccagcaagtg gtttgcaata gtgtacctta aagatattaa ctaatttatt 7800
agaagaccat ataaacagtc gagttgtcag aagtcgatag atactatcga ttgcaacgcc 7860
cggcgttatc gattgcaatc ggcttgcaat aaaaataatg attttttgat tataattttc 7920
agagattatt aaaaaatatt ttaaattttt taaaattata tatttagcaa ttaaagaaag 7980
tcatgcaaag acatgaggaa tgtccccaag ttgccaatag gcgattgttt cgccagttca 8040
ttggccacac tggtcaccag ctgaaaacac aaaaaccgat cgtacagcat aaatttagct 8100
cgaaaatgga ctaaacaaag acagcgatcc ggaatccgag cggaaacata gtctgcatga 8160
actatctaac gatcctgctg tgcaaccgaa aaccgacgat gctctcgcg cggacaaggg 8220
agaagtccca gcacaaggag ggcgtgggtg ggaagtacat gaagaaggac accccaccgg 8280
atatttcggg gatcaatgtg tggagcgatc agcgggcca gaagaaatcg ctgcagcgct 8340
gtgcgagcac ctgcgccagc tgcgagttcc atccgcgcag ctgcgagcac agtcggaaca 8400
cctactcctg cagcgactcg cagccggact actaccatgc tcgacgagca cagagccaga 8460
tgccctcgca gcagcactcc cactcgcatc ctcaactctt gccccacccc tccatccgc 8520
atgtgcgtag tcatcctccc ctgcgcgccc accagttccg cgccagcagc aatcagttga 8580
gtcagaacag cagcaactac gttaatttcg agcagatcga gcggatgcgc cgtcagcagt 8640
cgtcgccact gctgcagacc acatcatcgc cggcgccggg agccggagga ttccagcgca 8700
gctactccac caccagcgg cagcatcatc cccatctggg tggtgacagc tacgatgcag 8760
atcagggcct gctaagcgcc tcctatgcca acatgttgca actgccccag cggccacact 8820
cgcccgctca ctacgcgctc ccgcccagc agcagcagca tccacagatt catcaacagc 8880
acgcctcgac gccgtttggc tccacgctgc ggttcgatcg agctgccatg tccatcaggg 8940
agcgacagcc caggtatcag ccaactaggt aaactgcctc ttgaagtact atatttgaat 9000
agatagcgcg cgattgataa agtgggtaga gataatatga gcagctcttg attaaaggaa 9060
taatccgtaa aaactacata ttgtcaaaaa gtgcttaata ttattataac ttttaacaa 9120
tgacaatgca cgaaatgttt tattttcgaa acatttattg ttcaaagatt ttttatttga 9180
taacagattg ctttattttat ttacaataag aaaagttgat gtacaaaacc ggtttctact 9240
cgccttacaa taattaaaac aataacacaa tatatgattt tctgtacgag gaatataatg 9300
gaatatatat gatataatac acatttttaa acacattttc tcttctgttt ccacagctct 9360
ccgatgcagc agcaacaaca acaacaacaa cagcagcagc agcagctgca gcacacacaa 9420
ctggcagctc acctgggagg cagctactcc agcgattcgt acccgatcta cgagaatccg 9480
tcccgcgctc tctcgatgcg cgccacgcag tgcgagcgat cggagtgcgc catctacagc 9540
aatacgacgg cctcgtcggc cacgctggcc gtggttcgcg agcatcatca tcagggtcac 9600
ctggcggtgc catctggaag cgggggagga tccctgagcg gcagcggtcg tggtggcagt 9660
tctggcagtg ttcgcggegc ctctacctca gtgcaatcac tgtacgtccc accgcgaact 9720
ccgccagtg cggttgcgg agcgggaggc agtgccaatg ggtcgtcgca gaaggtagca 9780
tcacagcaat cgctcacgga gcccgaggag ctgcctctgc cgcccggctg ggccactcag 9840
tacacgctac acggtcggaa atactatatt gatcacaatg cgcataccac gcaactggaat 9900
catccgttgg agcgcgaagg tctgcgggtg ggctggcggc ggggtggtgc caagatgcat 9960
ggcacctact atgagaacca gtataccggg cagagccaac gtcagcatcc atgcttgacc 10020
tcctactatg tctacacgac gtctgcggag ccaccgaaag cgattcgacc agaggcgctc 10080
ctctatgccc caccacgca cactcacaat gcactgggtg cggccaatcc ctatctgctc 10140
gaggagatcc ccaagtgggt ggccgtctac tcggaggcgg actcgtccaa ggaccacctg 10200
ctgcagttca acatgtttag cctgcgggag ctggagggtc tcgacagcat gctgggtcgg 10260
ctcttcaagc aggaactggg caccatcgtg ggcttctacg agcgctaccg gtaagtgagc 10320
ggccacatgc cgctgcattc tccgtctccc gaaaagccac tactctcttg ttacaccttt 10380
cagtcgcgct ttgatactcg agaagaatcg acgcgcgggc cagaaccaga accaaaacca 10440
gtgaccgggt gaccaggtga cgactgactc agaccacata ctgcgcagca gctatatgca 10500

```

```

catcatagtg ctctgtaat cgacctttaa cttattttaac catcgactca tcgcgaaatc 10560
agtgccttat acgaaaccag acgagatggt agccaagcag atccatgaca gttcgaatgc 10620
cttgatgaaa cgtagaattg tgctacgttc tatataacct taatgtgatt tgagcttggc 10680
gtttgtttgt aatgtgagca aagaaaatta aactggttta ctgatcatct tacctgccga 10740
gcgcaattgt aatcgatgtg ccacctgaaa cccacaggt atttaacctg ggagtcggat 10800
tcatcgacgg atgttttggg aattcagcgc cgcgaagtgt aaataaaggg caacagttgg 10860
tggccaagtc ttactcgact tggcttggca catatttccg agttccatgc caagttttcg 10920
attcgcttgc aaaaattatg cattgggcac aagtgaatcg tggccgattc tgtattggca 10980
aaaaaaaaaa cagcgctcca atagaaagtg aatcttatgt ttgttttcgt ttggctatgc 11040
ttatttttag tcgaacctga taattcattc agtcgcctct tatcgaatgc ttataaaact 11100
ttatagtcac tgtttctgca ggtccctcaa aaacagtttc tactgctgat aagaagtttt 11160
cgaagtctgg ggagtattcg gcattggaaa ggccaaaagt tgtgttttat tatattttga 11220
acatattaaa caggatacat aaaacgagag ttttagattg taattacatt tgcataatct 11280
tttgctaaat tgataagtaa acagaaaata tgactcgatg gatattattg actaataata 11340
tatatttagg ggtttggtat gattactttg tactgtgaga tacaagttcg tttgtccac 11400
agatactttt caattcatag cttatctac agatacattt caattcatag cttatcccg 11460
agatacattt ccattcattg cttatccac agatacattt tagcatattt tttttgaaat 11520
ttgaatttga aaaaaaagtg tttttttttt ttttgttttg agaactactc gtcttgtcaa 11580
aatattttaac tgttcccgac tgaagtgcc accttttcgg ccgccgggtt ctcaagtgca 11640
aaaataatgt ataataaaaa gccaaagatac gtcggcggtc cgctctcgcc ccacttgttg 11700
ttgctgctgc cgctggtgcg tcgctgccgc tgccgcagtc gacgtcgact ccactgctcc 11760
aatattttaa cggatccatt ggatcgcgca ctcagtcgca ctggagagtc gccatcgag 11820
ccatcatcat agcattccat tccacttgta gccatcgga gtcgctcaat cgtcagttgg 11880
gacacattat ttaacttcat tottaacgtg agtgaattga tgtgttgggt ggcatcatg 11940
catatagcat aggcaaacaa ctgttcta at ccgcattatc ttaatcaca taatccggcg 12000
gcttatacag atgttttgcg ttagcagttg gcggtctaaa gcctctgctt gccacatgc 12060
cagtgaagt tctaaccgg ctcaaacaga cgcacaacaa gcgtatctcg tgcgtggaat 12120
catgaatgaa taaatgggtg ttactgttaa ctaacaatgg acctttttac caatcaatcg 12180
tcttatctat caccagaatt gaaacagaat tagtgaataa cttatggtgc atatcagttg 12240
aaacatgaag attcgtgtga acgatcgtga aagatatggt gttcgaactt taaattacc 12300
ttgtagttta ccactctcat tagttttgat ttatgtagaa ccaaaatttg gatcgtgact 12360
tgcgattagt attgcaatcg cagtgcattg cccaatctat tgattatctg caacttggtg 12420
cagactgccg caataattcg acggacacta tcagctagct ccattgattg agataagccc 12480
gttctcacgc ggtgttttac acttcttggc aatcgccaag tcacggccct cgccatataa 12540
aaaatatagt atgaacaatc gggaatcttt tggttttacg atcgaccgac aaagcccag 12600
tatttcctgt tacgtccatt tgggccatat aggcacataa aatgggtgct ccaacgcttg 12660
ccgtgggaaa gtgtgctcca attgcaaagt tgtaacattg agcgacattt gatgaagggt 12720
accgactttt atctcgacaa aaacacacac gaattccaga tgaagcgagc gtgcgtagtt 12780
tgactgcaa gttttttttt tggacaaat agttttatgt ttatatcatt ttatatcata 12840
ttatattcct tattgattga gtgtctgcac gggtcattaa attaagaagc aaaaaaaaaa 12900
aagggtgtcag gaattgcatt ccatactcct acgagtagat atcaatttca cccgatcgtg 12960
gtcaattggt caattgaagt aattcacaat tgaatcaata caataccata tagggcttca 13020
ttgaagaaga tgccagcagg actggatgct catgcatgaa taagttgaac gttgaacgca 13080
agcagaatgg atttcagcac acaccgctg accactttgc tgctcctcct cctggccaca 13140
ggtgagatat cgcaatccag atattgcat ctaataatga gggaatttct cctgcccaca 13200
gttgccctgg gaaatgccc aagcagtcag ctcaccgtcg attcccatga catcacggtt 13260
ctgctgaaca gcaacgagac tttcttggtg ttcgccaaagt gagttgccat tgccgggaaa 13320
tccaaatcca aaacatatgg catcgtaaat ctattgtgcc cattacagcg gattgctaga 13380

```

cagcgacgtg gaagttgctg tgggaacaga ttoggaggat catttgctcc tcgatcccg 13440
aacgtttgtg tatccagcgg gcagtactcg aaatcagtcg gtggtgataa ctggcctcaa 13500
agccggcaac gtcaaagtgg tcgcagatag cgatgatgcg aacaaagaga tgtgagtaac 13560
ttcacgggaa tcccaactgt tcccgtaact aattggaaaa ttcaacttatt ttccagtggtg 13620
aaggatgtgt tcgtacgcgt gactgtggcc aaatcgagag ctttgatcta cacctccatc 13680
atctttggct gggtttactt tgtggcctgg tcggtgtcct tctatccgca gatctggagc 13740
aactatcgcc gcaagtccgt cgagggaactg aactttgatt tcctggccct caatatcggtg 13800
ggcttcaccc tgtacagcat gttcaactgc ggccctctatt tcacgagga tctgcagaac 13860
gagtagcagg tgcgatatcc gctgggagtg aatcctgtga tgctcaacga cgtggtcttc 13920
tcaactgcgt ccatgttcgc cacctgcatt acgatccttc agtgcttttt ctatcaggta 13980
ataatatata tagcaaatac cattcaatag ccttatcgcc gaagtggcaa cagttgtcgc 14040
attgaacact aattgccatc aatcaaatg ccaaatcatt tgaatcacag cggatagtta 14100
cgatatgaag agtagataag gttttgactt gtaaaacatc catactttgt taaatttgtc 14160
cagagagcac agcaaagggg gtcgttcatt gcctacggaa tattggccat ctccgccgtg 14220
gtggtcgtcg tgtctgccgg tttggccgga ggatccgtca tccattggct ggactttctg 14280
tactactgca gttacgtcaa gctaaccatt accatcatca agtacgtgcc gcaagctctg 14340
atgaactatc gccggaagag cacctccggc tggagcatcg gcaacattct gctggatttc 14400
acgggaggaa cgctgagcat gctgcaaatg attctgaatg ctcataatta cggtaggata 14460
tagtctatca atttgtgatt ttccaatgaa atcgtgtctg gtttccagat gattgggtgt 14520
cgattttcgg tgatcccacc aaattccggc tgggtctgtt ttccgtgctc ttcatgtgt 14580
tcttcatgct gcagcactat gtgttttaca ggtgattgaa acattgtgtg aatatgatac 14640
ttaatctacg attatgtcat ctccactgta cacttatcat tattgtgtg ctgttttcca 14700
tttctcccca ggcattcgag ggaatcctcg agctctgacc tcaccaccgt gaccgatgtt 14760
caaaatcgaa caaatgagtc gccgccgccg agcgaagtga cgactgagaa atattagagc 14820
tgcattatca tatgtctgct gtagagaaag acttttgtgc cagtagcgct ttatgtacat 14880
ttttagaatt gtaaatatat ccgtatgccg tagctgccta agctttgtat aattcgtgcg 14940
ttttaattga aatttagttt gactaaaatt tgggaatttca ccattaaata aaacttaatt 15000
ttttgtagga gccagaaatc atacggtaca ttgctcgacc attcaaaggg ctgtgcagtg 15060
aaaccaattt gctgcatacg gcgcgttatt tgcaaaactaa taaatagatt gaagtattga 15120
aaaaatttca aaacagaaat tctaacttgc cgcacaatgg gcagcactgt tcgcactcgg 15180
ccaaatcctt atcgatagct tatcgatagc catggatata tgacattaag ttagccaatt 15240
tccggttagt tgacatccct ggagcacgga agattcttgc ggacacaaat cgcaactgct 15300
aaataaaatt tatttatttg agtgcacagc catgagtcct cacaagtcgg cgtcgttttag 15360
cttgactttt aaccagttag cgagatatt ttattcggtc ttaccaaca aaataatgtt 15420
gcgccttttt gcagaaacac ttcgattgtt tcgcgtagca atagtcgcac aatttttgaa 15480
gctttcaagg agttcctgga tttttgggat atcggcaacg aagtttctgc agagtcagca 15540
gttcgggtct ccagcaacgg agctttcaac ttgccgcaga gttttggcaa cgaatccaac 15600
gaatatgccc acctggctac gcctgtggat ccagcctacg gaggcaacaa cacgaacaac 15660
atgatgcagt tcacgaacaa tctggaaatt ttggccaaca ataattccga tggcaataac 15720
aaaattaatg catgcaacaa attcgtctgc cacaaggggt gagcaaatc aaaacacgcg 15780
ctccaatcga taaacattgg ctacggcgat tgctcgcgt gcgtggcgaa tggcaaaatc 15840
caaatagtcg gtggccacta cgattctgta gttttttgtt agcgaatttt taatatattag 15900
cctccttccc caacaagatc gcttgatcag atatagccga ctaagatgta tatatcacag 15960
ccaatgtcgt ggcaaaaga aaggtacagt gcggcaacaa attgatgac gaacagtaga 16020
aaccttgcat gtagcaacac gcttgtaact gcacattcg cgcggccaac ttgtttgtgt 16080
ttgtttatcc agccaaggcg cagtttgcca ctaagttttt atttcccttt tacactttag 16140
cactgattcc gaggatgact ccacggagggt cgatatcaag gaggatattc cgaaaacggt 16200
ggaggtatcg ggatcggaat tgtgagtacc tggtcacgtg gtcacatgtg gtttgcctgg 16260

```

ttgctaacta ttattgtttt tattattoca ggaccacgga acccatggcc ttcttgacagg 16320
gattaaacgt gagttgtgct tttaatgtgc aaagctatag cttactaact atttaaatatt 16380
attccccgca gtccgggaat ctgatgcagt tcagccaggt gggtaacatc gattagctat 16440
tgcattctga agcgtcgga cagatcgcc tgcacgagga tcagcaggaa gctggccacc 16500
gccgagaaga cattgctgat cagtcgcatg tccagctcgt acaagcccaa ggggtttaatt 16560
tggtacttg taccgtgac cagcagagta aagccgtgga ctgcctgacg gtagcggctg 16620
tccgcatgct ggagattcat ctctggaga atgactgccg atcttcgggt ggccaccaat 16680
aggtggttgc acaaatgcgt gagcaatgtg atctccgcca gcgagatgga gaggaaaacc 16740
agattgatca gcgatccaag accatcgta cggcttgccca tgattaaggt gtccgctatg 16800
gcatagtaca gactgtagaa acccaccgtt attccgagca ggtggcatat gagcgacaga 16860
atcatggaca aggacattgg ggtcagatac tttcccgaat gcacatatat caacctatag 16920
cgatacgcca gctggctgag ttcacccgcc aaggcgcaaa atcgctgcat gcggtagtat 16980
ttagtgtaga acttttagctg gtccctcctc tgcagcagat tcacctcctg cagctgacct 17040
tccagccgtc tggtcagagc gtacagaatc tccttcacca ccaccattgc gccaaagtag 17100
cagttattga gaaaattcga aataattaag ggaaacagcc ggtacaaggt ccagatcaag 17160
ctcatctcgg gatgctgccg cctctgttgc agtatgaaag ccacttcaat tgtagagga 17220
aaagccacgg tcttgaccag agccaaaacg atggatatgt acagcgacct gctgtccaga 17280
cggaattctt ttagggatc aaagaagggc actttgtcga acaccttggc cacatggtca 17340
ctgattatca tttgcgacac atagttaata acagccaccg taatgttcat atagctgtac 17400
agagtgggtg cgtccttcag gttgatctga ccctcctgg actccttgta gatttgccgc 17460
ccgtaaacca agctgaatgc aattgccac agcgaagcaa aggccagatt tgcctttgag 17520
aagcgaatc tttcacgacg gcccgccga tatcgattgg ccaggagtcc gaagacggtc 17580
ataaagccta tcagtatgat cgtcagaaat ttcaccatac gccgatgcgc gtagtcgctg 17640
gtgaagtcca tttctctcga acaattaata caaactgtga gcgcacttcc cacagcatta 17700
atatctgctt aattgttttc caactaccca actgatgcca tctagaggac ctgtcaagta 17760
gccggacact atcgggacac atcgcgaaac gcatgtattt caccggccgt ccagaaacca 17820
actgagcatg cgttgtgcta ctactagcca caaacaaga agcataagaa gcgtgagga 17880
agcggcattc cttgcgtgac tcagccgctg cctgcaattt cataagagcg acatgacgtc 17940
aaagtcgctt cgaagttcac tttcagttgg aggacagaac aaaacactct tatctagccg 18000
attagcacgg tgcactcctt cccgtcgtca tegttagcg agaatttcaa gcacttgtga 18060
aaaatagaat agaatacaaa acaaatcgcc agtccatttg taactcgagc aagctggaac 18120
atgaagctct atcagctcta tgagcgcaaa gtgtgaaccc ttatatgatt gcgagttaag 18180
ttgacattca aataatatct tgtttttgct tacagcaatc cgtgctgcgc gaaatgatgc 18240
tgcaggacat tcagatccag gcgaacacgc tgcccaagct agagaatcac aacatcggtg 18300
gttattgctt cagcatggtt ctggatgagc cggccaaagtc tctttggatg tactcgattc 18360
cgctgaacaa gctctacatc cggatgaaca aggccttcaa cgtggacggt cagttcaagt 18420
ctaaaatgcc catccaacca cttaatttgc gtgtgttccct ttgcttctcc aatgatgtga 18480
gtgctcccgt ggtccgctgt caaaatcacc ttagcgttga gccttgtaag tgaagataac 18540
aatacagatc gaacaggatt atttaactat catttgtaca aacctttagt gacggccaat 18600
aacgcaaaaa tgcgcgagag cttgctgcgc agcgagaatc ccaacagtgt atattgtgga 18660
aatgctcagg gcaaggggaat ttccgagcgt ttttccgttg tagtccccct gaacatgagc 18720
cggctctgtaa cccgcagtggt gctcacgcgc cagaccctgg ccttcaagtt cgtctgcaa 18780
aactcgtgta tggggcgaaa agaaacttcc ttagtcttct gcctggagaa agcatggtaa 18840
ggtgacagca aaactctaga tggctagaac aaagcttaac gtgttttctt tcttgacgcg 18900
gcgatatcgt gggacagcat gttatacatg taaaaatatg tacgtgcccc aagcgggac 18960
gcatccaaga cgaacgccag ctcaatagca agaagcgcaa gtccgtgccg gaagccgccg 19020
aagaagatga gccgtccaag gtgcgtcggg gcattgctat aaagacggag gacacggaga 19080
gcaatgatag ccgagactgc gacgactccg ccgcagagtg gaacgtgtcg cggacaccgg 19140

```

atggcgatta cegtcctggct attacgtgcc ccaataagga atggctgctg cagagcatcg 19200
 agggcatgat taaggaggcg gcggctgaag tcctgcgcaa tcccaaccaa gagaatctac 19260
 gtcgccatgc caacaaattg ctgagcctta agagtaagca gtgaatcgga ggacaaagag 19320
 attaagcttt acttaccgaa ctttcctttc agaacgtgcc tacgagctgc catgactttc 19380
 gatctggctg acaatctccc aggtatcaga tacctttgaa atgtgttgca tctgtggggg 19440
 atactacata gctattagta tcttaagttt gtattagtcc ttgttcgtaa ggcgtttaac 19500
 ggtgatattc cccttttggc atgttcgatg gccgaaaaga aaacattttt atatttttga 19560
 tagtatactg ttgttaactg cagttctatg tgactacgta acttttgtct accacaacaa 19620
 acatactctg tacaataaag ccaaaagtga atttattaaa gagttgtcat attttgcaaa 19680
 catatcctcg tgggtgtacgc caatgccag agcctactgt acccccaccg tggagcacat 19740
 gctatgtgac atgtgtggct tgtgtgcggc caatgcactc aggatgcaac tcagctagct 19800
 agctgcta atgtcaaaat tgctgcgtcg cttttacata ctttatttat acccgatatc 19860
 gcacgtcttt ggttttagtt ctatgctttc aaaaaaaaaa aaacaacctc aagcagggcg 19920
 catgcgttgc gccagcgttg cacatgtgcg aggatgcaaa aaagtgcac aaacaccaga 19980
 tgttgacact gtgccgctgc agctgcaggc gacttttagct tttgccacat gcggcagcta 20040
 aatgtttact ctagcccacc gatcgctgtt cattgacctc gggcaggggc attaagtgcg 20100
 ccctaactcg aacggaatga tagcctctgt gtccaaaaat tcagccaaag cggatgcact 20160
 cacttccatt tggggcctgt ccttcttcga ccggctgcca cttccactac cagtttgga 20220
 ccacgaaaat gggctgttca aagtgtcaa aaccagcgg agcaactcac tcaattctcg 20280
 ttggacgagc gcacagaaaa gtggttttgg atacgagttg agttcgagag acctttctgc 20340
 actgggaaca tacatgcggc tttgtgtaac agaataataa agtacgcaa catatctgta 20400
 atacttaaag cacaagaac aaatataaat gtatcataat ttgtttaatt atttattcga 20460
 ggtttccaaa caagtcattc tgataacaaa agttgtaaaa ataaaaacca ctaaaattaa 20520
 atatcaccca cttctcagaa taagcacagc tgtatatact tcagtatata tttttttcag 20580
 tgcacttttc ccaagcgatg caatcgccctt agaagcccaa ttaaatacgt ttctttgatt 20640
 ggcgggtgcc aaaaggttga caattcgaaa gtggcgacaca ctgggaggca gtgactcata 20700
 atttacataa ttatttcggg aagatattaa gactcatact atattcaagc agttgtttat 20760
 cattttaaac tggcagatac cccatcttta cggaccagat aaagggaaag caaacacggc 20820
 tgggctctta tcggctacga tottcatccg cagttcccac tgtgcgcgtg gggaaaacaa 20880
 tatggcccaa acacataaaa aacaacaaaa aaaggaaaca accacagaaa gccgggcta 20940
 gacgtcaggt gaaacgcagt agcttccactc gcgactcggc gcttccactc aaagggtgcta 21000
 ccgtgccc caataatctg cagctcgtag atacgaaaac cagatagcgt cgagcggctg 21060
 gcgatcttca ctcaatgggg ggaaatactg ctatagagtc gaaagcttgt acacgtagtt 21120
 tggcattcgc agtcgcttgt tggcgttttt agtctgctgc ctgatcttcg acgcgtgca 21180
 gctgttttgg agtcgccg agtgccatat ttgctttgac cgcgaaaatt tctgggcta 21240
 aaacagagat atttgagata cagatacata tatctcatat cacatattag ccaattgtgg 21300
 gtgcaacaag ctgtgagtga tgggtggagac ggcaacgaca acgaccataa cccgcaccac 21360
 caccgccgtt ccggctgggt cagtaacggt aacaggaccc actgcctcgg ccacgcccac 21420
 cgcgacacag gcggccgcgc aggcgcactc caacgatgag accaccggg ccactttcaa 21480
 tctgaaagtc atcgtctttc tgctcctcct gcctctggtc ctgctggccg tctttctcaa 21540
 gcacctgttg gattacctat tcgcgctggg actcaaggag aaggatgtca gtggcaagg 21600
 ggcactggtg agttgcattc gagtgcccat tggggctaac aaatggctgc aatgagcgtc 21660
 tggcaaatga gccattaata aggctagtca gatgcacatc agacatggat gcacttagaa 21720
 aatgcagtcg catttcatgt taagtactga cattaaaaaa gagatatatg tctgtgttta 21780
 gatacatctt tgggtaccaaa attaggttca gatacttcgt aaagaaattg gtaatggtat 21840
 actttaatcg ttggcttcat gtgaatttgt tttcccagta tccgcttcta agtgatcttg 21900
 tatctgacga ctacttagcc aaccagaaac gtcacgcact ttccttttcc agtggctgcc 21960
 tccgggtttc caccacgccc acctttggct caccacctt tcccccttc cgccttttct 22020

```

ttgcttttta tttctcctct tttttttttt tttgatgtca ctgccattag ggtgcggtcg 22080
atcgcttagt actgtgttat taatgtaa atttatgcgt ttggtgcca gcttggttag 22140
ttggtggcca attgttttagt tgtgtccaca gagccgcgtc ttggtgcca cggacagtta 22200
atgtgacata atttcgctgt aagcgcgtgca atcaaagtga atctccagct gaaatcgtgc 22260
tcatggcaac catatcgcg cccaataatc acatatgcat cttggggcgt cgaattatgg 22320
agaagtcaat tgccaatggg cgccaatgcc actggacaag gtcaagtgat gatgccgctg 22380
ccgatgctcc atatcgtaaa gaacctgac gaattcggaa cccattagca tgcttttcag 22440
gctttttata gtgggcgtgt gccggccata agcgtctcac gtagcgtatt aatgattcac 22500
agcggcccgga cttttgtttt agtctcagct ttttttttcg atcgttccct cagatatcgt 22560
tttctcagat acagatacac atacagatac atttttgttg cggttgcaca gtggtatttt 22620
cgggtggcag ggactggaga attcccatgc caactgttag cagcaactta attataagat 22680
tgactttcgt tgataagttc tattgacatc atggttgccg aattcgagtt atttcagctc 22740
aaaaataccc cttttttcga caccactggc caacggccaa ctgcaaactg gttttgcgtg 22800
tgctgcctata tttattttcca agatgaacga aaagagcgca aaaatgcaaa cctcagaaag 22860
ttcacttttg ttttcagtct aatgtttgtg tttacaaaca atagagtgtga gaatttcgat 22920
gggccc aaagt atctgcaagt gtgtagcatg cggggtatct ctcatagtcg tagataaaac 22980
tcaactactg ttgccgctgt taatttgc atgatattga aattcttcgg ctgttctata 23040
atcacaacaa ctgcgcattt gttattgttt tccccattgc tagtcgctaa cgtgccc aac 23100
tctgaattga actcattccg gcttacattt cgattcacc aactaccgca cacc aaaaac 23160
ggcggctgag gtcacccagt gggcttcaat tacgggtcaaa agtcactcaa ttgtgccc a 23220
gagggctcgg ccaccgagcg tatgagtaat gccattcata agtcgcctct gccgctgttg 23280
ctgctgctca cataattgtc cgtaaatgag gtttttgttc aatgcgaagt cacattagct 23340
cgagttgatt gtttgcaaat taagctaatt aatttacttg agtatacgag tgtaatgtga 23400
gtaacctgtg atttaaacc aggtgaccgg cggagggcgt gggctgggtc gcgagatctg 23460
cttggaactg gcgcggcggg gctgcaagct ggcgcgtcgt gatgtcaact ccaagggatg 23520
ttacgaaacg gtggagctgc tctccaagat tccacgctgc gttgccaaagg cctacaagg 23580
gagttcacta gctgcttgga tatttaatgg tttgataaca agaactctta ttccagaacg 23640
acgtgtcatc gcctcgcgag cttcaactga tggccgcca ggtggagaag gaactgggtc 23700
ccgtggacat tctgggtcaac aatgcctccc tcatgccc atgactcaaca cccagtc tga 23760
agagcgatga aatcgacaca atactgcagc tcaatctggg ctctacata atgggtgagtg 23820
tgtgcttctg aaaatgggac aaatataaaa cttcttgatt ttgcagacca ccaaggagtt 23880
cctgccgaag atgataaacc gcaagtccgg tcatctggtg gcagtaaatg ccttagcggg 23940
taagcttact tggttaaagt gcttaccact tcattgatac ctatgtatat ataactcgca 24000
tttaggtcta gttccactgc caggagcggg catctacacg gccaccaa atcggaatcga 24060
gggcttcatg gaatcgctgc gagctgagct gcgattgtcc gactgtgact acgttcgcac 24120
cacgggtggc aatgcctatc tgatgaggac cagcggagat cttccactgc tcagtgatgc 24180
ggggtaagat tggtttatag tttgggcaga tcacttggtc tcatgcggt actacattta 24240
gcattgccaa gagctatccc ggactgccc caccatattg ggccgagaag attgtcaagg 24300
gcgtgttgct gaacgagcgc atggtgtatg tgccaaaa attcgcactc agtgtatggc 24360
tgctcaggtg agaattgaat tagcccaggt aaccagcgat tatttctaac gattattgtt 24420
gtcgccttgc tttagactgt tgcccaccaa gtggcaggat tacatgctgc ttcgcttcta 24480
ccacttcgat gtgcgcagct cccacctgtt ttactggaag tagggcacag gagaaggcac 24540
atccccacc agaagcattt actcctgttt gtttcccaat tgcagttctt tattcaactg 24600
ttgcttacgc tagtgtaca tgtttagcta tttatacgaa tctttaactt aaattaaatc 24660
tatatcctaa cattagaatt acgtccgggt ggcctttcct attttatttc gtataagccg 24720
aagttgttcg gagtagcaca tctctcggga ctgctggacg caggacctcc gttcgtagt 24780
ccaagtgtag ttcaagtggc atcgatggac cagcttgagg ccaactggagc agtagtagaa 24840
gtaggcgcag ttccgtggat gtggcataaa gccatagact ccctcctggc agttgatgat 24900

```



```

attctctcgc gtttgcacgc gattgcagga cactagatga gcaggagtagc aggccttggc 24960
cagtecagcc ccctcgtagc agaccatata aggataacat ggtccggcat tgggtaaaag 25020
tcgcagggtg atcgccaatg gttccgcttt ctgagctggc ttcttgacca tcgaggggga 25080
tttagtggtt atgcctacgg gatcccgga tctcgacacc aactttcgat ccaaacagcg 25140
ttccaatttt tcgtcgtagt aatgaccatc caagcactcg gcctcaaagg atcctggacc 25200
ggcacaatat atgtatttgg agcaattgct agagctggcg acataaactc ccaattgtgg 25260
agcactggca cactcttcga actccagggc actggatcga tgaccagca aggtcaccaa 25320
aataattgtt aagaaggtta cagctcccat ttcatttatt tttttaacga ccgaaatagc 25380
gggatgactt ctgtagactg acttcacga tgatgggttg agtatatttt tgcattgtgt 25440
ccaactgata aagaagacaa gttattccat cgattactac gctgggtatc gtctggtaga 25500
taccgcta at gagcacatgg cagtaactgc cacgcccact ctgggcgggtc tcggtaattt 25560
gcattttcgt agcatacttc gcagcagcag caaagcaacc gagtatttaa tgataccaca 25620
ccgcagcata atgctcgact gggcgccggt tcaataaaaa ttgaaaatgc actcaattcg 25680
caattaagtg tcgccacttc cgtacggaca agcggacaaa cggacggaca agcggacaaa 25740
tggacggata aacggacgga tggatggctg tcgaacgata ccattcaggc cattcaatcc 25800
attcatcgca gtcatcctca ttattatttc catcgctcgc gtggtcgttg ctggctggag 25860
ttaagcgatg gccatcgatt taatatccga tgagatatcc ataacttgca attaggtttg 25920
gtggctctgc gctttacgta aatgattgct tagccgatta atgaagaatt accagtgcga 25980
atggctggga tctgtgggca ttatccaatt gaccaactac catgctaccc cactaccatt 26040
accattacca taatgtgcaa tgtgccaat gggctcaaat taaaagtttt attaatgtc 26100
aattaaacgc tgcgcccag cagctgcttt gtggcataat ttttgggtca atctgcatat 26160
ctgattaaca ggttataccg ctccagtctac tacatatacc atgcaccaga tgcgcgggg 26220
cacagacaac aagaagtaaa agaaaggacc ccatatggtg ccgacggctc aagtgattaa 26280
gtgcacgacg agatcttcaa atgcagtga acatgtgcac aaatacaaaa cacacacaca 26340
cacacacaca cagcatatt gaaaatgtat gtaaattcta attagattg tggatgaaga 26400
ccccagcac cttgatactt ctgctcaatg cgcattgctc atgctcagcc ccgcatccga 26460
agatccataa aaatagctca ctaattattt gtgtgctagg gttacagttc tcataaaaaa 26520
caaacaaact gtcgggcgtt ttatggatct tctgcctcta tggcctcaat gcccccgca 26580
agttttcgat cccattcga ttcgaaaccg aagaagagct acgaccaatc acttttcaat 26640
tcctatgagc agttgagcat caattgattt cgatatgaaa ataaaaataca tttatttatt 26700
atcacattac gtatcacagc cattcgcccg cctacgccct ggcatctgga tcgccacatc 26760
catcgtgctg accttggtgc ggcatttccg agctgattag cctccgaatc tcgaccagaa 26820
cccggtccgt tcgagcctcc aggttgctga gggcggtgtt taggtcatcc aagctggaat 26880
tgactctggc catcagacgc tccgagttgt tggtcagctc gatgaggta tcgaaactgc 26940
tggcctggcg actctccatc gatatcctgt ccagatccag ctgcagctgc tcatcgccgc 27000
tgtccatctg ggctttaagg gctggaaaac aactttcgat ttaaatttaa atttttttca 27060
ccctaaatca tgattttcgg tgttattttg tgccatgca tccgaagtgt aaagcaaatt 27120
tgacttggtt tgttttgcta tcgaacataa ttaaagttgc ttaccataaa ccaatttaat 27180
ttaattgtaa ttgcagctaa ctggcttttg ggtacttttg cttttaacgc caaatgtgaa 27240
atattaagta tattttattt aagcgatggc acctgtaaat tgagatttaa gggggtatat 27300
taaattgggtg aacttgatga tttttttttt tcatcaaacg tttattaaag tctattgctt 27360
aaaaaatga aagtaaattg cttgccattt taggaggata tttttgaaaa atcgttacaa 27420
ctttt 27425

```

<210> 19

<211> 1781

<212> DNA

<213> *Drosophila melanogaster*

<400> 19

```

gaattcggca cgagacgccca tacaaaaagt tgggaactgag tgggaatcgga gtactatata 60
gccagccgat cccttccaga gcgccggaag agtagctcac atccgaaccc acgtccccga 120
gccgatgtcg cggcggggaat agagcgattc gcagtcctaaa cagcatgata aacccattg 180
catccgagtc ggaggccatc aattcggcca cctatgtgga caactatata gattcgggtg 240
aaaatctgcc ggacgacgtg cagcgccagt tgtcacgcat ccgcgacata gacgtccagt 300
acagaggcct cattcgcgac gtagaccact actacgacct gtatctgtcc ctgcagaact 360
ccgcggatgc cggcgacggt tctcgaagca tctccaggat gcaccagagt ctcatcagg 420
cgcaggaact gggcgacgaa aaaatgcaga tcgtcaatca tatgcaggag ataatcgacg 480
gcaagctgcy ccagctggac accgaccagc agaacctgga cctgaaggag gaccgcgac 540
ggtatgcgct cctggacgat ggcacgcctt cgaagctgca acgcctgcag agcccgatga 600
gggagcaggg caaccaagcg ggcactggca acggtggcct aaatggaaac ggcctgcttt 660
cggccaaaga tctgtacgcc ttgggcggct atgcagggtg tgttgtgcct gggttctaat 720
ccatgacctc cggcaacggt ggcggctcaa cgcctcaactc ggagcgctcg agccatgtca 780
gtaatggtg caacagcgcc tccaatggca atgccagcgg cggaggaggc ggagaactgc 840
agcgacaggg tagcaagcgg tcgaggaggc gaaacgagag tgttgttaac aacggaagct 900
ctctggagat gggcggaac gagtccaact cggcaaatga agccagtggc agtgggtggtg 960
gcagtggcga gcgcaaatcc tcgttgggcy gtgccagtgg agcgggacag ggacgaaagg 1020
ccagtctgca gtcggcttct ggcagtttgg ctagcggctc tgcagccacg agcagtggag 1080
cagccggagg tgggtggtgcc aacggagccg gcgtagtgtg tggcaataat tccggcaaga 1140
agaaaaagcg caaggtacgc ggttctgggg cttcaaatgc caatgccagt acgcgagagg 1200
agacgccgcc gccggagacc attgatccgg acgagccgac ctactgtgtc tgcaatcaga 1260
tctcctttg cgagatgatc ctgtgcgaca atgacctgtg ccccatcgag tggttccatt 1320
tttctgtcgt ctccctggta ctaaaaccaa aaggcaagtg gttctgcccc aactgccgcy 1380
gagaacggcc aaatgtaatg aaacccaagg cgcagttcct caaagaactg gagcgctaca 1440
acaaggaaaa ggaggagaag acctagtcta ttaggcagc ctatccaacc cattgctctg 1500
tgtctaacac caggctctgt aaaatattcg atcctaagat ttaccttaat gtatatttag 1560
tgactttctt agaccgcgac ccttttcgac tttccctctt ttcaccagc ttagatccct 1620
cgcttctatg gttataggtc gtcagttttc atttaaagtt tctgtacaaa caatatcttt 1680
ctcaatgtaa acacacaaaa actcgtataa ttagagtaca cctaaactta atttatggta 1740
ataaacggtg atattcaaaa aaaaaaaaaa aaaaaactcg a 1781

```

<210> 20

<211> 433

<212> PRT

<213> *Drosophila melanogaster*

<400> 20

```

Met Ile Asn Pro Ile Ala Ser Glu Ser Glu Ala Ile Asn Ser Ala Thr
  1             5             10            15

Tyr Val Asp Asn Tyr Ile Asp Ser Val Glu Asn Leu Pro Asp Asp Val
      20             25            30

Gln Arg Gln Leu Ser Arg Ile Arg Asp Ile Asp Val Gln Tyr Arg Gly

```

35	40	45
Leu Ile Arg Asp Val Asp His Tyr Tyr Asp Leu Tyr Leu Ser Leu Gln		
50	55	60
Asn Ser Ala Asp Ala Gly Arg Arg Ser Arg Ser Ile Ser Arg Met His		
65	70	75
Gln Ser Leu Ile Gln Ala Gln Glu Leu Gly Asp Glu Lys Met Gln Ile		
85	90	95
Val Asn His Met Gln Glu Ile Ile Asp Gly Lys Leu Arg Gln Leu Asp		
100	105	110
Thr Asp Gln Gln Asn Leu Asp Leu Lys Glu Asp Arg Asp Arg Tyr Ala		
115	120	125
Leu Leu Asp Asp Gly Thr Pro Ser Lys Leu Gln Arg Leu Gln Ser Pro		
130	135	140
Met Arg Glu Gln Gly Asn Gln Ala Gly Thr Gly Asn Gly Gly Leu Asn		
145	150	155
Gly Asn Gly Leu Leu Ser Ala Lys Asp Leu Tyr Ala Leu Gly Gly Tyr		
165	170	175
Ala Gly Gly Val Val Pro Gly Ser Asn Ala Met Thr Ser Gly Asn Gly		
180	185	190
Gly Gly Ser Thr Pro Asn Ser Glu Arg Ser Ser His Val Ser Asn Gly		
195	200	205
Gly Asn Ser Gly Ser Asn Gly Asn Ala Ser Gly Gly Gly Gly Gly Glu		
210	215	220
Leu Gln Arg Thr Gly Ser Lys Arg Ser Arg Arg Arg Asn Glu Ser Val		
225	230	235
Val Asn Asn Gly Ser Ser Leu Glu Met Gly Gly Asn Glu Ser Asn Ser		
245	250	255
Ala Asn Glu Ala Ser Gly Ser Gly Gly Gly Ser Gly Glu Arg Lys Ser		
260	265	270
Ser Leu Gly Gly Ala Ser Gly Ala Gly Gln Gly Arg Lys Ala Ser Leu		
275	280	285
Gln Ser Ala Ser Gly Ser Leu Ala Ser Gly Ser Ala Ala Thr Ser Ser		

290 295 300
 Gly Ala Ala Gly Gly Gly Gly Ala Asn Gly Ala Gly Val Val Gly Gly
 305 310 315 320
 Asn Asn Ser Gly Lys Lys Lys Lys Arg Lys Val Arg Gly Ser Gly Ala
 325 330 335
 Ser Asn Ala Asn Ala Ser Thr Arg Glu Glu Thr Pro Pro Pro Glu Thr
 340 345 350
 Ile Asp Pro Asp Glu Pro Thr Tyr Cys Val Cys Asn Gln Ile Ser Phe
 355 360 365
 Gly Glu Met Ile Leu Cys Asp Asn Asp Leu Cys Pro Ile Glu Trp Phe
 370 375 380
 His Phe Ser Cys Val Ser Leu Val Leu Lys Pro Lys Gly Lys Trp Phe
 385 390 395 400
 Cys Pro Asn Cys Arg Gly Glu Arg Pro Asn Val Met Lys Pro Lys Ala
 405 410 415
 Gln Phe Leu Lys Glu Leu Glu Arg Tyr Asn Lys Glu Lys Glu Glu Lys
 420 425 430

Thr

<210> 21

<211> 2666

<212> DNA

<213> Drosophila melanogaster

<400> 21

cattttgtac agtctaaaacg gggattcgcg taaactacgc agaaatataa acaaacaaaa 60
 actagtagac tatagaatat aaacagtttc ctaccaatgg agacttgtga agtggaggga 120
 gaggcggaga cgctggtgag acgcttctcc gtcagctgcg agcaattgga gctggaagcg 180
 agaattcagc aaagcgctct gtccacctac catcgcttgg atgcggtcaa cgggctgtcc 240
 accagcgagg cagatgccca ggagtggctg tgttgccgct tctacagcga actgcagcgc 300
 tcgaagatgc gcgatattag ggagtccatc aacgaggcaa acgattcggt ggccaagaac 360
 tgctgctgga acgtgtcact aaccgctctg ctgctgcagct ttaagatgaa cgtgtcccag 420
 tttctacgcc gcatggagca ctggaattgg ctgacccaaa acgagaacac tttccagctg 480
 gaggttgagg aactgcgttg tcgacttggg attacttcca cgctgctgcg gcattataag 540
 cacatctttc ggagcctgtt cgttcaccgc gcaaggggtc ggaccgggt gccgcgaatc 600
 actaccaagc gctgtatgag ttcggttggg tgctcttcct ggtcattcgc aacgagttac 660
 ccggttttgc gattacaaac ctgatcaacg gctgtcaggt gctcgtttgc acaatggatc 720

```

tccttttcgt gaacgcctta gaggtgcccc gatccgtagt tatccgcgcg gagttctctg 780
gagtgcccaa gaattgggac accgaagact tcaatcctat tttgctaaat aaatatagcg 840
tgctagaagc actgggagaa ctgattcccc agtaccagc gaagggagtg gtgcaaatga 900
agaacgcctt tttccacaaa gccttaataa tgctctatat ggaccatagt ctagttggag 960
acgacaccca tatgcgggag atcattaagg agggatgtct agatatcaat ctggaaaact 1020
taaatcgcaa atacaccaat caagtagccg acattagtga gatggacgag cgtgtgctgc 1080
tcagcgtcca gggggcgata gagaccaaag gggactctcc taaaagccca cagctcgcct 1140
tccaaacaag ctgcgtcacct tcgcatagga agctgtccac ccatgatcta ccagcaagtc 1200
ttcccctaag cattataaaa gcattcccca agaaggaaga cgcagataaa attgtaaatt 1260
atntagatca aactctggaa gaaatgaatc ggacctttac catggccgtg aaagattttt 1320
tggatgctaa gttgtctgga aaacgattcc gccaggccag aggcctttac taaaaatatt 1380
tgcagaaaat tttgggaccg gagctggttc aaaaaccaca gctgaagatt ggtcagttaa 1440
tgaagcagcg caagcttacc gccgccctgt tagcttgctg cctggaactg gcacttcacg 1500
tccaccacaa actagtggaa ggccctaagg ttccttttgt cctgcaactg ttttcaactg 1560
acgcctacga ctttcaaaaag attctagagt tgggtggtgc ctacgatcat ggttttcttg 1620
gcagagagct gatcaagcac ctggatgtgg tggaggaaat gtgcctggag tcgttgattt 1680
tccgcaagag ctcacagctg tgggtgggagc taaatcaaag acttccccgc tacaaggaag 1740
tcgatgcaga aacagaagac aaggagaact tttcaacagg ctcaagcatc tgccttcgaa 1800
agttctacgg actggccaac cggcggtgc tccttctgtg taagagtctt tgcctcgtgg 1860
attcctttcc ccaaatatgg cacctggcgc agcactctt caccttagag agtagccgtc 1920
tgctccgcaa tcgacacctg gaccaactgc tgttgtgcgc catacatctt catgttcggc 1980
tcgagaagct tcacctcact ttcagcatga ttatccagca ctatcgccga cagccgcact 2040
ttcggagaag cgcttaccga gaggttagct tgggcaatgg tcagaccgct gatattatca 2100
ctttctacaa cagtgtgtat gtccaaagta tgggcaacta tggccgccac ctggagtgtg 2160
cgcaaacacg caagtcactg gaagaatcac agagtagcgt tggatattctg acggaaaaca 2220
acttccaacg aattgagcat gagagccaac atcagcatat cttcaccgcc ccctcccagg 2280
gtatgcaaaa gtggctcctg ctccagtcac ccaccttcat ctcgccgcgc atcaccactt 2340
tccttgcaaa gctcgcccaa cgtaaagcgt gctgcttcga gtaacgactt gatgagagag 2400
atcaagcgac caaacatcct gcggcgctgc cagctttcag tgatctaata accaatcaaa 2460
aaaggcttaa atacttggct gcattttacg cagctagctt agtatatttc ttaaaactcaa 2520
aaatggtaat taaataatgt ttaaattata gatattttat taacttgttc aagtaagtta 2580
aaagcttttg cttttgtaaa aataaaggaa taactgccac tcgtagttta aataaatttt 2640
taaaaaaaaa aaaaaaaaaa ctcgag 2666

```

<210> 22

<211> 556

<212> PRT

<213> *Drosophila melanogaster*

<400> 22

Met Asp Leu Leu Phe Val Asn Ala Leu Glu Val Pro Arg Ser Val Val

1

5

10

15

Ile Arg Arg Glu Phe Ser Gly Val Pro Lys Asn Trp Asp Thr Glu Asp

20

25

30

Phe Asn Pro Ile Leu Leu Asn Lys Tyr Ser Val Leu Glu Ala Leu Gly

35	40	45
Glu Leu Ile Pro Glu Leu Pro Ala Lys Gly Val Val Gln Met Lys Asn		
50	55	60
Ala Phe Phe His Lys Ala Leu Ile Met Leu Tyr Met Asp His Ser Leu		
65	70	75
Val Gly Asp Asp Thr His Met Arg Glu Ile Ile Lys Glu Gly Met Leu		
	85	90
Asp Ile Asn Leu Glu Asn Leu Asn Arg Lys Tyr Thr Asn Gln Val Ala		
	100	105
Asp Ile Ser Glu Met Asp Glu Arg Val Leu Leu Ser Val Gln Gly Ala		
	115	120
Ile Glu Thr Lys Gly Asp Ser Pro Lys Ser Pro Gln Leu Ala Phe Gln		
	130	135
Thr Ser Ser Ser Pro Ser His Arg Lys Leu Ser Thr His Asp Leu Pro		
	145	150
Ala Ser Leu Pro Leu Ser Ile Ile Lys Ala Phe Pro Lys Lys Glu Asp		
	165	170
Ala Asp Lys Ile Val Asn Tyr Leu Asp Gln Thr Leu Glu Glu Met Asn		
	180	185
Arg Thr Phe Thr Met Ala Val Lys Asp Phe Leu Asp Ala Lys Leu Ser		
	195	200
Gly Lys Arg Phe Arg Gln Ala Arg Gly Leu Tyr Tyr Lys Tyr Leu Gln		
	210	215
Lys Ile Leu Gly Pro Glu Leu Val Gln Lys Pro Gln Leu Lys Ile Gly		
	225	230
Gln Leu Met Lys Gln Arg Lys Leu Thr Ala Ala Leu Leu Ala Cys Cys		
	245	250
Leu Glu Leu Ala Leu His Val His His Lys Leu Val Glu Gly Leu Arg		
	260	265
Phe Pro Phe Val Leu His Cys Phe Ser Leu Asp Ala Tyr Asp Phe Gln		
	275	280
Lys Ile Leu Glu Leu Val Val Arg Tyr Asp His Gly Phe Leu Gly Arg		

290	295	300
Glu Leu Ile Lys His Leu Asp Val Val Glu Glu Met Cys Leu Glu Ser		
305	310	315 320
Leu Ile Phe Arg Lys Ser Ser Gln Leu Trp Trp Glu Leu Asn Gln Arg		
	325	330 335
Leu Pro Arg Tyr Lys Glu Val Asp Ala Glu Thr Glu Asp Lys Glu Asn		
	340	345 350
Phe Ser Thr Gly Ser Ser Ile Cys Leu Arg Lys Phe Tyr Gly Leu Ala		
	355	360 365
Asn Arg Arg Leu Leu Leu Leu Cys Lys Ser Leu Cys Leu Val Asp Ser		
	370	375 380
Phe Pro Gln Ile Trp His Leu Ala Glu His Ser Phe Thr Leu Glu Ser		
	385	390 395 400
Ser Arg Leu Leu Arg Asn Arg His Leu Asp Gln Leu Leu Leu Cys Ala		
	405	410 415
Ile His Leu His Val Arg Leu Glu Lys Leu His Leu Thr Phe Ser Met		
	420	425 430
Ile Ile Gln His Tyr Arg Arg Gln Pro His Phe Arg Arg Ser Ala Tyr		
	435	440 445
Arg Glu Val Ser Leu Gly Asn Gly Gln Thr Ala Asp Ile Ile Thr Phe		
	450	455 460
Tyr Asn Ser Val Tyr Val Gln Ser Met Gly Asn Tyr Gly Arg His Leu		
	465	470 475 480
Glu Cys Ala Gln Thr Arg Lys Ser Leu Glu Glu Ser Gln Ser Ser Val		
	485	490 495
Gly Ile Leu Thr Glu Asn Asn Phe Gln Arg Ile Glu His Glu Ser Gln		
	500	505 510
His Gln His Ile Phe Thr Ala Pro Ser Gln Gly Met Pro Lys Trp Leu		
	515	520 525
Leu Leu Gln Ser Ser Thr Phe Ile Ser Arg Arg Ile Thr Thr Phe Leu		
	530	535 540
Ala Lys Leu Ala Gln Arg Lys Ala Cys Cys Phe Glu		

545

550

555

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/06602

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C07K 14/00; C12N 15/00

US CL : 435/455; 530/350; 536/23.5; 800/3, 13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455; 530/350; 536/23.5; 800/3, 13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST

Lialog (file: medicine)

search terms: p53, Rb, tumor suppressor, Drosophila, insect.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DONEHOWER et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 19 March 1992, Vol. 356, pages 215-221, entire document.	1-28
A	FIELDS et al. Presence of a potent transcription activating sequence in the p53 protein. Science. 31 August 1990, Vol. 249, pages 1046-1049, entire document.	1-28
A	KUSSIE et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science. 08 November 1996, Vol. 274, pages 948-953, entire document.	1-28

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 JUNE 2000

Date of mailing of the international search report

09 AUG 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ANNE-MARIE BAKER, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06602

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEVINE, A. J. p53, the cellular gatekeeper for growth and division. Cell. 07 February 1997, Vol. 88, pages 323-331, entire document.	1-28
A	RAYCROFT et al. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science. 31 August 1990, Vol. 249, pages 1049-1051, entire document.	1-28